PHOTOTROPHIC BACTERIA GROWN IN PALM OIL MILL EFFLUENT AS FEED FOR CULTURING ROTIFERS AND MARBLE GOBY LARVAE

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ABSTRACT

The culture of the highly priced marble goby *Oxyeleotris marmorata* (Bleeker) in the Asian region is currently beset by the poor supply of fingerlings which are largely collected from the wild, while larviculture based on conventional feeds has not been producing enough due to high larval mortality. Phototrophic bacteria (PB), an unconventional feed has not been seriously considered as an alternative feed despite being nutrient-rich. Thus, the overall objective of this study was to evaluate the feasibility and success of culturing larval marble goby and rotifer (*Brachionus rotundiformis*) using a selected PB species grown in palm oil mill effluent (POME) as feed. The PB species tested were *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides* and *Rhodovulum sulfidophilum* (PD1). Live feed (rotifers and *Artemia* nauplii) cultured from the settled biomass of PB produced in synthetic 112 medium could not support the survival of larval marble goby. Only live feed fed the biomass of POME-grown *R. sulfidophilum* (bPOME-PD1) supported good larval survival. Fatty acid analysis revealed that only bPOME-PD1 contained both docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The study supports the hypothesis that PB grown in synthetic media lacks the essential fatty acids or/and their precursors that are crucial for larval fish survival. The effective use of bPOME-PD1 as a feed for marble goby larvae, given directly or indirectly (via live feed), has demonstrated that larvae fed directly could not achieve consistent survival, whereas those fed indirectly showed consistent and significantly higher ($P<0.01$) survival. It may be that bPOME-PD1 is not of optimal prey size or/and it contained unsuitable high ratio of DHA/EPA and ARA (arachidonic acid)/EPA. The batch culture of rotifers using unsettled POME-grown *R. sulfidophilum* culture (cPOME-PD1) supported even higher rotifer production (898 individuals/mL) as compared to bPOME-PD1 (323 individuals/mL) or just POME (533 individuals/mL). Fish larvae survived better in 5 ppt salinity as compared to 10 ppt

salinity. The increase in stocking density from 10 to 15 larvae/L did not affect the larval survival and growth, but higher stocking density of 20 larvae/L and 30 larvae/L resulted in low survival and growth. Larvae cultured in grey coloured tanks and given live feed fed with cPOME-PD1 had the highest survival as compared to those cultured in black and transparent tanks. The findings did not totally accept the hypothesis that the dark coloured tank supports higher survival of marble goby larvae than light coloured tank. Further investigation using grey tanks demonstrated that live feed fed cPOME-PD1 or bPOME-PD1 were equally good in sustaining good larval fish survival (71.4 - 81.9%) and growth (11.07 - 12.30mm total length). Surviving larvae contained low ratios of DHA/EPA (3 - 4) and ARA/EPA (0.1 - 2) as compared to larvae fed bPOME-PD1 or microalgae (*Nannochloropsis* sp.) which contained high ratios of DHA/EPA (>11) and ARA/EPA (>5). The optimal conditions for marble goby production are grey tank, 5 ppt salinity, larval stocking density of 15/L and live feed fed POME-PD1 to give DHA:EPA:ARA ratio of approximately 7:2:1. A stable marble goby larviculture with viable commercial production has resulted from this study.

ABSTRAK

Kebelakangan ini, penternakan ikan ketutu *Oxyeleotris marmorata* (Bleeker) yang bernilai tinggi di rantau Asia telah mengalami kemerosotan disebabkan kekurangan pembekalan anak-anak ikan ketutu yang mana kebanyakannya diperolehi daripada habital asal, sebaliknya larvikultur berasaskan makanan konvensional tidak mampu dihasilkan dengan secukupnya kerana kematian larva yang tinggi. Fototrofik bakteria (PB) yang merupakan makanan bukan konvensional pula kurang dipertimbangkan sebagai makanan alternatif walaupun ia kaya dengan nutrien. Justeru, objektif keseluruhan kajian ini adalah untuk menilai kebolehlaksanaan dan kejayaan dalam penternakan larva ikan ketutu dan rotifer (*Brachionus rotundiformis*) dengan menggunakan spesis PB terpilih sebagai makanan yang mana spesis ini dikultur di dalam air kumbahan kilang kelapa sawit (POME). Spesis PB yang digunakan ialah *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides* and *Rhodovulum sulfidophilum* (PD1). Makanan hidup (rotifer dan *Artemia* nauplii) yang dibiak daripada biojisim PB yang dihasilkan di media sintetik 112 tidak berupaya menampung kemandirian larva ikan ketutu. Hanya makanan hidup yang diberi biojisim *R. sulfidophilum* yang dikultur di POME (bPOME-PD1) memberi kemandirian larva yang baik. Analisa asid-asid lemak menunjukkan hanya bPOME-PD1 mempunyai DHA (docosahexaenoic acid) dan EPA (eicosapentaenoic acid). Kajian ini menyokong hipotesis bahawa PB yang dikultur di media sintetik kekurangan asid-asid lemak perlu atau/dan prekursornya yang penting untuk kemandirian larva ikan. Keberkesanan penggunaan bPOME-PD1 sebagai makanan untuk larva ikan ketutu yang diberi secara terus atau tidak terus (melalui makanan hidup) menunjukkan larva yang diberi biojisim PB tidak mampu mencapai kemandirian yang konsisten, sebaliknya larva yang diberi makanan hidup menunjukkan konsistensi dan signifikan tinggi ($P<0.01$) terhadap kemandirian. Ini menunjukkan bahawa bPOME-PD1 berkemungkinan bukan dalam saiz optima makanan atau/dan ia

mengandungi ketidaksesuian kadar DHA/EPA dan ARA (arachidonic acid)/EPA yang tinggi. Penternakan rotifer secara kelompok menggunakan kultur *R. sulfidophilum* yang dibiak di POME (cPOME-PD1) menyokong penghasilan rotifer dengan lebih tinggi (898 rotifer/mL) berbanding dengan bPOME-PD1 (323 rotifer/mL) atau POME sahaja (533 rotifer/mL). Larva ikan dapat hidup dengan lebih baik di kemasinan 5 ppt berbanding kemasinan 10 ppt. Peningkatan stok densiti dari 10 ke 15 larva/L tidak menjejaskan kemandirian dan pertumbuhan larva tetapi stok densiti yang lebih tinggi, 20 larva/L dan 30 larva/L telah mengakibatkan kemandirian dan pertumbuhan yang rendah. Larva yang diternak di tangki berwarna kelabu dan dibela dengan makanan hidup yang diberi cPOME-PD1 mempamerkan kemandirian tertinggi berbanding dengan larva yang diternak di tangki berwarna hitam dan lutsinar. Penemuan ini tidak menyokong sepenuhnya hipotesis di mana tangki yang berwarna gelap memberi kemandirian yang lebih tinggi berbanding dengan tangki berwarna cerah. Eksperimen selanjutnya menggunakan tangki kelabu menunjukkan makanan hidup yang diberi cPOME-PD1 atau bPOME-PD1 adalah setanding dalam menampung kemandirian (71.4 – 81.9%) dan pertumbuhan (11.07 – 12.30mm jumlah panjang) larva ikan yang baik. Larva-larva ini mempunyai kadar DHA/EPA (3 – 4) dan ARA/EPA (0.1 – 2) yang rendah berbanding dengan larva-larva yang diberi bPOME-PD1 atau mikroalga (*Nannochloropsis* sp.) yang mengandungi kadar DHA/EPA (>11) dan ARA/EPA (>5) yang tinggi. Kondisi optima untuk pengeluaran ikan ketutu adalah tangki kelabu, kemasinan 5 ppt, larva stok berdensiti 15/L dan makanan hidup yang diberi POME-PD1 pada kadar DHA:EPA:ARA bersamaan 7:2:1. Kajian ini telah menunjukkan potensi penghasilan larvikultur ikan ketutu yang stabil secara komersil.

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LIST OF SYMBOLS AND ABBREVIATIONS

ARA	arachidonic acid (C20:4n-6)
B1	*Rhodopseudomonas palustris*
BOD	biochemical oxygen demand
bPOME-B1	biomass of *Rhodopseudomonas palustris* grown in palm oil mill effluent
bPOME-KS	biomass of *Rhodobacter sphaeroides* grown in palm oil mill effluent
bPOME-PD1	biomass of *Rhodovulum sulfidophilum* grown in palm oil mill effluent
b112-B1	biomass of *Rhodopseudomonas palustris* grown in 112 medium
b112-KS	biomass of *Rhodobacter sphaeroides* grown in 112 medium
b112-PD1	biomass of *Rhodovulum sulfidophilum* grown in 112 medium
ca.	approximately
cm	centimeter
COD	chemical oxygen demand
cPOME-PD1	culture of *Rhodovulum sulfidophilum* grown in palm oil mill effluent (unsettled)
C18:3n-6	β-linolenic acid
d	day
DAH	day after hatching
DHA	docosahexaenoic acid (C22:6n-3)
DO	dissolved oxygen
dph	day post-hatch
DW	dry weight
EFA	essential fatty acids
e.g.	for example
EPA	eicosapentaenoic acid (C20:5n-3)
et al.	and other people
etc.	and other things
EUS	epizootic ulcerative syndrome
FAME	fatty acid methyl esters

FAO	Food and Agriculture Organisation
F1	*Filial 1*
g	gram
g	standard gravity
g/L	gram/litre
h	hour
HAF	hour after fertilization
HCG	human chorionic gonadotropin
HUFA	highly unsaturated fatty acids
i.e.	that is
ind/mL	individuals/millilitre
kg	kilogram
KS	*Rhodobacter sphaeroides*
L	litre
LA	linoleic acid (C18:2n-6c)
LNA	α-Linolenic acid (C18:3n-3)
LT	long-term
Lux	Luminous emittance and illuminance
mg	milligram
mg/g	milligram/gram
mg/kg	milligram/kilogram
mg/L	milligram/litre
min	minute
mL	millilitre
mm	millimeter
MUFA	monounsaturated fatty acids
NaCl	natrium chloride
NH_3	unionized ammonia
no.	number
OA	oleic acid (C18:1n-9c)
OD	optical density

PB	phototrophic bacteria
PCA	principal components analysis
PD1	*Rhodovulum sulfidophilum*
PG	pituitary glands
PNSB	purple non sulfur bacteria
POME	palm oil mill effluent
POME-B1	*Rhodopseudomonas palustris* grown in palm oil mill effluent
POME-KS	*Rhodobacter sphaeroides* grown in palm oil mill effluent
POME-PD1	*Rhodovulum sulfidophilum* grown in palm oil mill effluent
POS	palm oil sludge
ppm	part per million
ppt	part per thousand
psi	pound-force per square inch
PUFA	polyunsaturated fatty acids
r	population growth rate
SFA	saturated fatty acids
SGR	specific growth rate
SO	specific objective
sp.	species
ST	short-term
TL	total length
vs	versus
v/v	volume/volume
w/w	weight/weight
112-B1	*Rhodopseudomonas palustris* grown in 112 medium
112-KS	*Rhodobacter sphaeroides* grown in 112 medium
112-PD1	*Rhodovulum sulfidophilum* grown in 112 medium
µm	micrometer
%	percentage
Δ	delta
<	less than

>	more than
=	equal to
℃	degree celcius

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 General introduction

The aquaculture industry will continue to expand and develop in most regions of the world due to the depletion of wild fish stocks and high demand for aquatic foods. In 2009, aquaculture accounted for 37.97% (55.1 million tonnes) of the total world fisheries (145.1 million tonnes) and the global aquaculture production is expected to increase in the years to come (FAO, 2010). Under the Third National Agriculture Policy or NAP3 (1998 - 2010) (Ministry of Agriculture Malaysia, 2000), the Malaysian government is also encouraging the aquaculture sector to fulfil the country's protein demands through increasing fish production instead of depending on capture fisheries (Galid, 2003; Sugiyama et al., 2004). In 2010, Department of Fisheries Malaysia reported that the freshwater aquaculture sector contributed 43.14% of the aquaculture production in Malaysia, while the brackishwater sector was 56.86%. According to various studies, marble goby *Oxyeleotris marmorata* (Bleeker) is the most favoured freshwater species due to its high commercial value with strong market demand (Cheah et al., 1994; Senoo et al., 1994a; Senoo et al., 1997; Luong et al., 2005). It is considered as a first grade fish in the Southeast Asian region due to its high protein and low fat content (Rakbankerd, 2005), lean, non-bony flesh and good taste (Mohsin & Ambak, 1983; Senoo et al., 1997; Lin & Kaewpaitoon, 2000; Amornsakun et al., 2003; Luong et al., 2005) and is also a good candidate species for research (Leatherland et al., 1990; Jow et al., 1999; Sayer, 2005; Masaya et al., 2006).

Marble goby is the world's largest tropical freshwater goby-like fish (Kottelat el al., 1993; Senoo et al., 1994a; Tavarutmaneegul & Lin, 1988) also known as "sand goby", and its local names include " ikan malas", "ikan ketutu", "ikan hantu", "ikan bodoh", "soon hock", "lam kor" and "bamboo fish" (Senoo et al., 1993a; Cheah et al.,

1994; Senoo et al., 1997). It can grow up to more than 2 kg in body weight and 50 cm in total length (TL) (Mohsin & Ambak, 1983; Roberts, 1989; Inger & Chin, 2002).

The marble goby larvae are still obtained from the wild due to the failure of mass larval culture in hatcheries. The production of marble goby via aquaculture was only 30.41 tonnes valued at RM 1.2 million (wholesale) (Department of Fisheries Malaysia, 2009). Other problems in marble goby culture include high mortality of larvae, disease, peculiar feeding behaviour and slow growth of fingerlings during nursing stages. According to Hoa and Yi (2007), it takes approximately one year for 5 g fingerlings to reach 50 g. As a result of the difficulties in rearing of marble goby larvae, the development of mass seed production has also been delayed. Thus, the understanding of its nutritional requirements is critical before developing a feeding regime because dietary nutrients influence larval survival and growth rate.

It was reported that the fatty acid composition of lipids found in marble goby is influenced by dietary inputs, and was shown that 16:0, 18:0, 16:1 and 18:1 are the predominant saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in marble goby (Nielsen et al., 2005). According to Ferreira et al., (2003), lipids are the major metabolic energy source in the majority of the fish species, given their central role as an energy source and essential fatty acids (EFA). Henderson and Tocher (1987) commented that the fatty acids in fish are derived either directly from dietary lipid or synthesised *de novo* from non-lipid precursors. The production of *de novo* fatty acid increases when diets contain high protein as protein is favoured as the carbon source for energy provision in fish (Sargent et al., 2002). Henderson (1996) reported that fish are unable to synthesize polyunsaturated fatty acids (PUFA – fatty acids with more than one double bond) completely *de novo* from non-lipid precursors. Freshwater fish are believed to have Δ (delta) 4, Δ5 and Δ6 desaturases (Henderson & Tocher, 1987) but lacked Δ12 desaturase enzyme which can desaturate OA (oleic acid, C18:1n-9c) to LA

(linoleic acid, C18:2n-6c) and the Δ15 desaturase acts by converting LA to LNA (α-linolenic acid, C18:3n-3). Therefore, these PUFA can only be obtained from the diet (Sargent et al., 2002). Further, freshwater fish commonly contain higher amount of n-6 PUFA, especially LA, and n-6 highly unsaturated fatty acids (HUFA - subsample of PUFA with ≥ 20 carbons), particularly ARA (arachidonic acid, C20:4n-6) and DPA (docosapentaenoic acid, C22:5n-6) compared to n-3 HUFA such as DHA (docosahexaenoic acid, C22:6n-3) and EPA (eicosapentaenoic acid, C20:5n-3) (Ackman, 1994). Bessonart et al., (1999) and Koven et al., (2001) reported that elevation of ARA could improve fish survival and growth as well as resistance to handling stress. On the other hand, most freshwater fish do not require long chain HUFA but often require PUFA such as LNA to synthesize EPA and DHA as well as LA to manufacture ARA (Henderson, 1996; Sargent et al., 2002). The ability of marble goby to bioconvert LNA to EPA and DHA as well as LA to ARA is unknown and requires further research.

Larval fish also have high demand for dietary protein mainly amino acids due to extensive catabolism of amino acids for production of metabolic energy and high growth rates (Rønnestad et al., 1999; Rønnestad et al., 2003). Larval survival and growth also indirectly depend on the success of the starter feed to maintain larval needs for amino acids (Srivastava et al., 2005). Therefore, introduction of these components (lipids and protein) into various vehicles such as phototrophic bacteria (PB) and zooplankton (rotifer; *Artemia* sp.) is important process in the field of larviculture (Coutteau & Sorgeloos, 1997; Dhert et al., 2001).

Rotifer is one of the most important primary feed of larval fish because of their amino acid composition and high digestibility (Hoff & Snell, 1997). *Brachionus* spp. are the most common rotifer species used to feed larval fish and are considered to be an essential food source for newly hatched fish larvae (Howell, 1973), even better than copepods (Theilacker & Kimball, 1984). However, *Brachionus* spp. are not considered

as complete feed because they lack n-3 HUFA which are essential for larval fish survival and development (Wilson, 1995; Sargent et al., 1997; Sargent et al., 1999a; Izquierdo et al., 2000). Their nutritional value can however be improved by feeding them with a highly nutritious diet since their biochemical composition is positively correlated to their diet (Lubzens et al., 1995). Conventionally, *Brachionus* spp. are fed with baker's yeast, microalgae or bacteria (Snell, 1991). Unconventional live microbial feed such as PB has not been seriously considered as an alternative aquaculture feed despite being nutrient-rich. Therefore, it is possible to manipulate the nutritional quality of rotifers by culturing them using PB. However, these PB appear to lack several EFA, particularly n-3 HUFA (Imhoff & Imhoff, 1995).

As fingerlings require fatty acids, in particular PUFA and HUFA, the enrichment of PB may be achieved by culturing them in palm oil mill effluent (POME). Although POME is a pollutant, it is rich in lipids, protein and carbohydrate. The recovery of POME nutrients has been demonstrated as useful for fish culture (Phang, 1990). This knowledge has contributed to the idea of culturing PB in POME so that the PB could be enriched with fatty acid nutrients from POME. The PB grown in POME will either be fed directly to larval marble goby or via a vehicle, such as rotifer and *Artemia* (Figure 1.1).

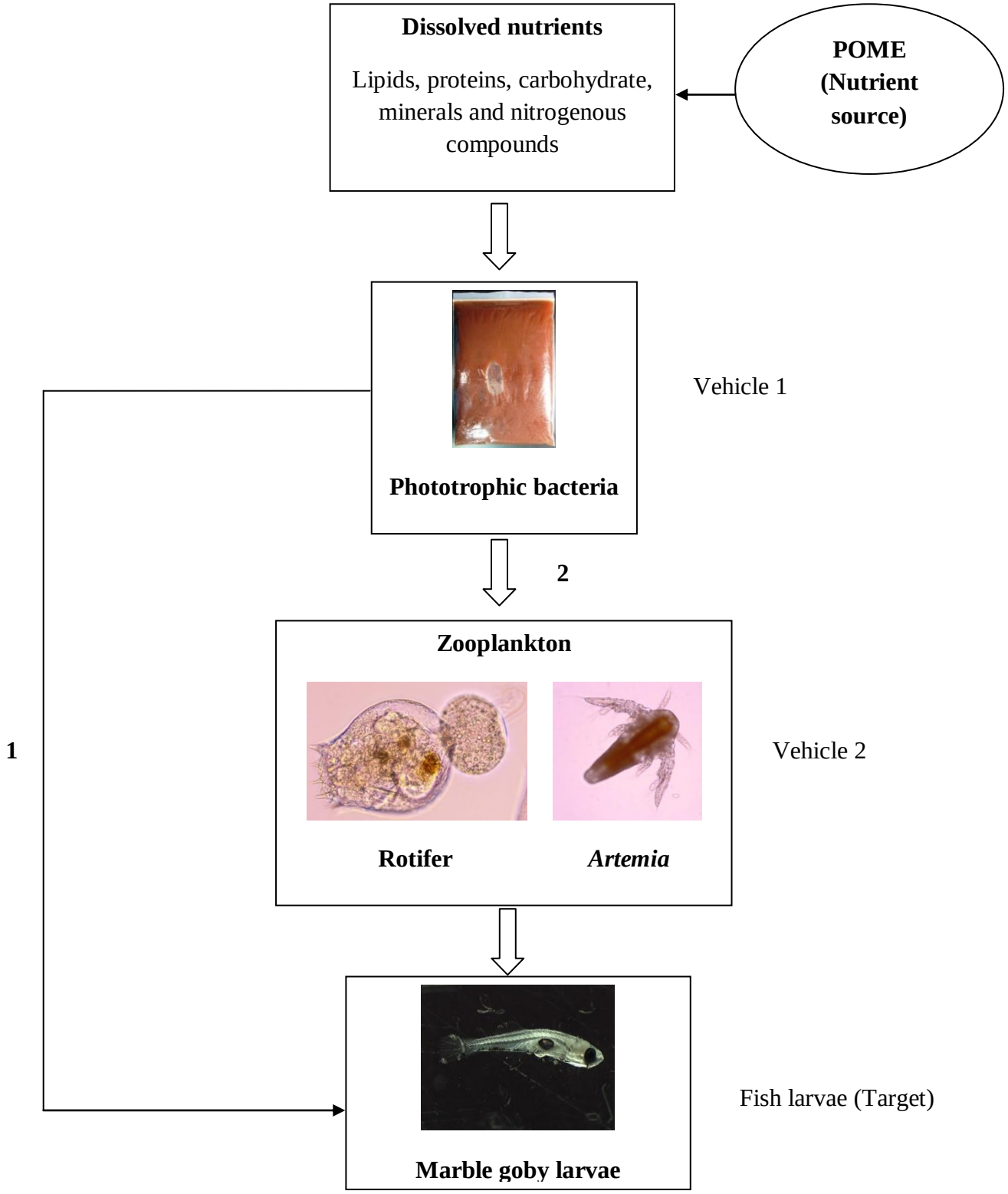


Figure 1.1 A conceptual model of present study to enrich live feed (bacteria, zooplankton) using palm oil mill effluent (POME) for marble goby culture. POME is used as a substrate for culturing phototrophic bacteria (PB). The POME nutrients are captured in the bacterial cells (vehicle 1) which are then directly fed to the marble goby larvae (Route 1) or via a second vehicle, zooplankton such as rotifer and *Artemia* (Route 2).

1.2 Problem statements

a) Aquaculture of high-value species of fish is currently beset by bottlenecks in mass culture. The current production of aquaculture food is now squaring off with the world's fish meal and fish oil production. The acute demand for such fish products stresses wild fish populations which are already overfished and competes with humans for similar products. This means that aquaculture nutritionists will have to find new sources of high protein and omega 3 rich oils.

b) The current production of valuable fish species such as the marble goby has been seriously constrained by the shortfall in fingerling supply. This is attributed to the low survival of hatchery produced larvae. For instance, the average survival of larval marble goby was 15.7% over 30 days of culture (Senoo et al., 2008). As a result, the grow-out or farming of this species relies heavily on wild-caught larvae or juveniles with serious environmental implication, including recruitment overfishing.

c) The palm oil industry is the largest agro-industry in Malaysia, contributing 3.3% of the national gross domestic product (GDP), but annually produces the largest volumes of fatty wastes in the form of POME. High in organic load, the POME pollutes the environment if discharged untreated.

1.3 Significance of the present study

The present research addressed the urgent need to reduce our present reliance on fish meals and oils by exploiting the versatility of PB to beneficially bioremediate polluting agro-wastewaters (like POME) and extracting its protein and lipid-rich biomass for the culture of commercially valuable species of fish.

The study is unique, in that PB is a yet unexploited, natural, indigenous and free-living bacterium that was used to feed traditionally used live feed such as rotifer and *Artemia*, to culture fish larvae. The PB itself will be enriched with essential fatty acids

(EFA) by culturing them in POME. Thus, this study will exploit readily available POME as a cheap source of nutrients for PB production. If the PB grown in POME is effective as feed for zooplankton and fish larvae, the PB grown in POME could be mass cultured and commercially produced. Thus, this is not only exploiting and treating a potentially limitless effluent resource but at the same time, converting it to valuable fish biomass cheaply. The potential conversion of POME to EFA by PB would thus open up a hitherto unused effluent of the palm oil industry for use in the aquaculture industry. Further, the development of an optimal feeding protocol based on enrichment of live feeds will benefit the aquaculture industry in the country.

1.4 Scope and objectives of study

The overall objective of this study was to evaluate the feasibility and success of culturing marble goby (*Oxyeleotris marmorata*) larvae and rotifers (*Brachionus rotundiformis*) using a selected species of PB (*Rhodovulum sulfidophilum*, PD1) grown in POME as feed.

1.4.1 Specific objectives

The specific objectives (SO) of the component study were:

SO1: To evaluate the benefits of biomass of PB grown in POME as direct feed for rotifer and indirect feed (via live feed such as rotifers and *Artemia* nauplii) for larval marble goby.

SO2: To optimize the conditions for growth of bacterial species, PD1 in POME (POME-PD1) in a selected reactor.

SO3: To investigate the production of rotifers using POME-PD1.

SO4: To evaluate the effectiveness of using biomass of PB grown in POME as a direct or indirect (via rotifers and *Artemia* nauplii) live microbial feed for rearing marble goby larvae in 5 ppt and 10 ppt salinity.

SO5: To investigate the effects of larval stocking density, tank colour and quality of live feed (rotifers and *Artemia* nauplii) on survival and growth of marble goby larvae.

1.4.2 Hypotheses

This study tested the following hypotheses:

a) PB grown in synthetic media lacks EFA or/and their precursors which are essential for larval survival. This hypothesis was proposed and tested in Chapter 3.

b) The dark coloured tank would support higher survival of marble goby larvae than the light coloured tank. This hypothesis was proposed and tested in Chapter 7.

1.4.3 Research approach

Figure 1.2 illustrates the research approach and studies taken to achieve the stated specific objectives.

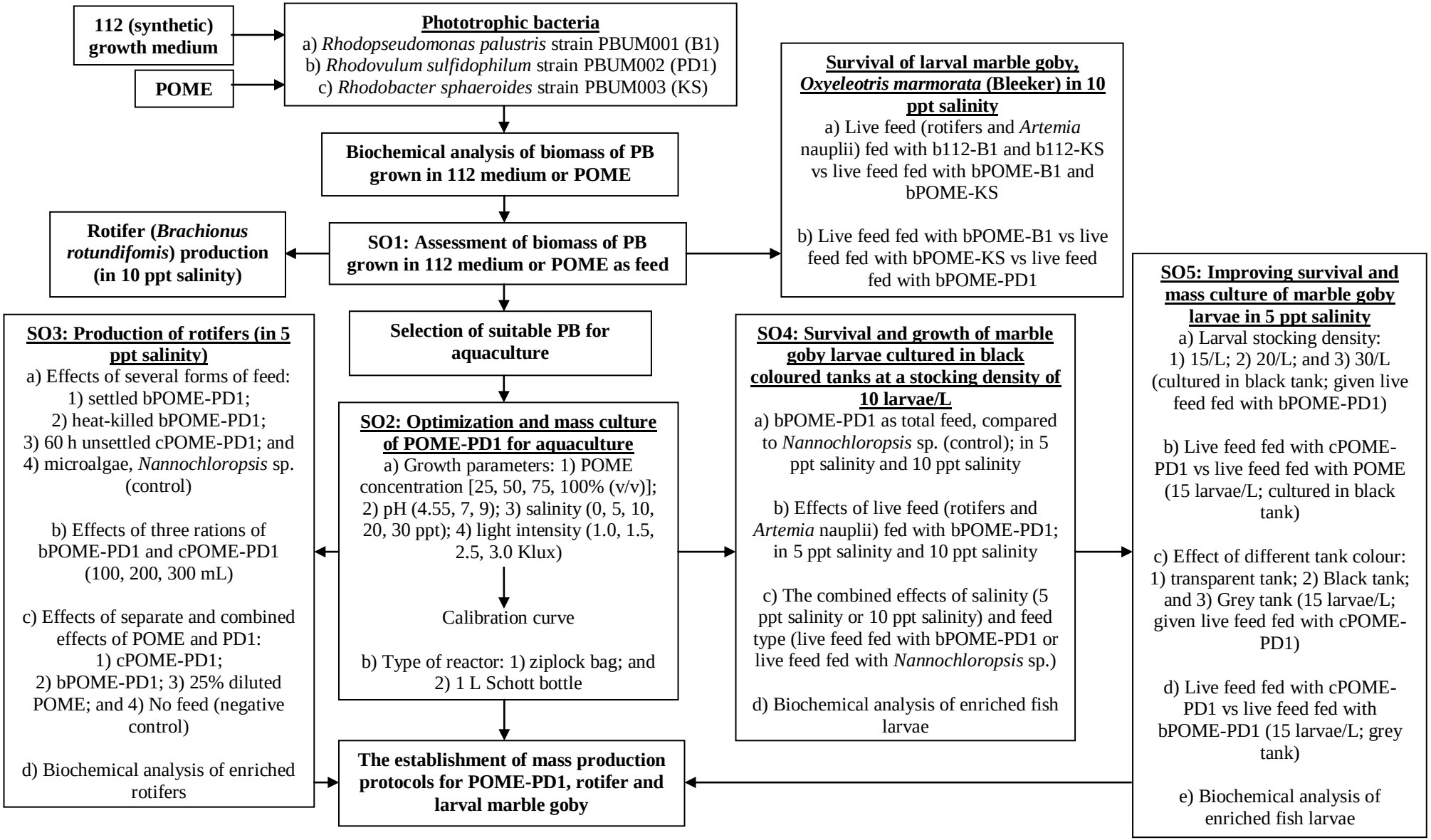


Figure 1.2 The research approach and studies taken to achieve the stated specific objectives (SO1 - SO5)

CHAPTER 2

LITERATURE REVIEW

2.1 Marble goby

2.1.1 Taxonomy and morphology

Marble goby *Oxyeleotris marmorata* (Bleeker) is a carnivorous freshwater fish, belonging to the Phylum Chordata, Family Eleotridae, Order Perciformes and Class Actinopterygii (Inger & Chin, 2002) (Figure 2.1). It is closely related to members of the family Gobiidae except that it has separate pelvic fins and six branchiotegal rays, whereas a true goby has united pelvic fins to form a cup-shaped sucker (Jayaram, 1999).

Figure 2.1 *Oxyeleotris marmorata* (weight: 366.8 g; total length: 29 cm)

The body is pale brown on the lower part and dark brown on the upper part with a series of large dark blotches. The black or dusty band fins have no spines. Further, the eyes are located on the flattened upper-side of the head and has a deep cleft mouth positioned upward (Kottlelat et al., 1993). The gender is distinguished based on their urogenital papilla where male fish has flattened and triangular shaped urogenital papilla, whereas female fish has reddish at the tip and barrel shaped urogenital papilla (Figure 2.2). The fish is also named ‘sleeper’ because of its habit of staying still in the bottom and wait for the prey (Jonna & Weinhermer, 2003).

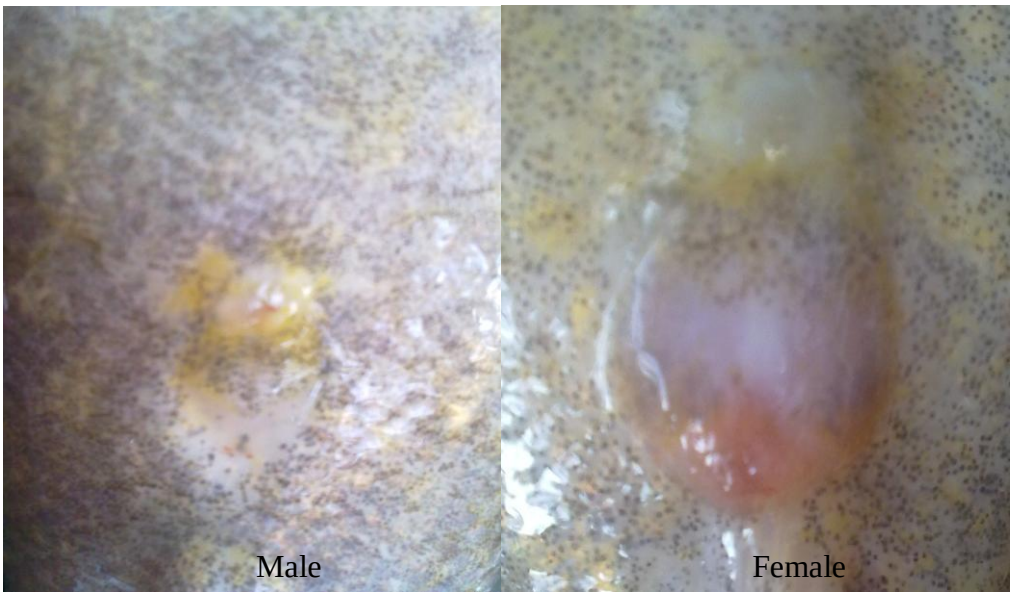


Figure 2.2 The urogenital papilla of male and female marble goby

The marble goby is commonly found in freshwater and brackishwater bodies throughout Southeast Asian regions such as Malaysia, Thailand, Brunei, Indonesia, Philippines, Fiji, Vietnam, Laos, Singapore and Cambodia (Sompong, 1980; Kottelat, 2001, Inger & Chin, 2002). It is well known to contain higher protein (15%) and low fat (0.2%) ratio than other commercially important carnivorous fish (Rakbankerd, 2005). The protein and fat contents are influenced by feed type (Jauncey, 1982; Ohta & Watanabe, 1996), feeding strategy (Jobling et al., 1998) and genetics (De-Santis & Dean, 2007).

2.1.2 Strategies of spawning

2.1.2.1 Natural reproductive behaviour

Marble goby is a multiple spawner which spawns throughout the year under optimum environment conditions (Tavarutmaneegul & Lin, 1988) with peak spawning occurring during the rainy season (May to August) in Thailand (Sompong, 1980). A mature female has relatively high fecundity that ranged from 130 to 300 eggs per gram body weight with more eggs produced in younger females (Pisarn & Wichaen, 1977;

CFRDB, 2006). In comparison, carnivorous freshwater snakeheads, *Ophiocephalus punctatus* and *Channa striata*, have average fecundity of 2 - 17 and 11 - 36 eggs per gram body weight, respectively (Kok, 1982; Ali, 1999).

The reproductive behaviour of male goby is size dependent. The larger male goby is a better nest builder and able to produce more milt as compared to a smaller male (Svensson et al., 1998). Generally, a female prefers a larger and colourful male for protection (Forsgren, 1997). Further, a female prefers a nest with the smallest entrance to mate and to reduce exposure of eggs to predators (Jones & Reynolds, 1999). After oviposition, the female will be chased out of the nest, while the male will remain to guard and incubate the eggs by fanning and brushing behaviour using its pectoral fins until hatching (Tan & Lam, 1973; Senoo et al., 1993b). Proper egg incubation tends to give a high hatching rate (Senoo et al., 1994a).

2.1.2.1.1 Induced spawning

Marble goby can spawn naturally throughout the year under proper feeding protocol and optimum environmental conditions. The spawning can be induced with or without hormone treatment. However, hormone injection to induce spawning has become a necessity in order to mass produce the larvae (Tavarutmaneegul & Lin, 1988). Commonly, Human Chorionic Gonadotropin (HCG) is preferred and more effective to stimulate the final maturation and ovulation in marble goby compared to acetone-dried common carp pituitary gland (PG) (Cheah et al., 1994). The hormone is injected either at the dorsal muscle (intra-muscular injection) or under the pectoral fin (intra-peritoneal injection) of the fish body. The former is recommended as it is safer and unlikely to damage vital organs as compared to the latter (Senoo et al., 1994a). The numbers of injections are dependent on the female maturity stage by indentifying their egg diameter and morphology. Polyethylene cannula is commonly used to sample the oocytes but is

only applicable for larger female with larger genital pore (Head et al., 1994). Injection of 3 IU (International Unit)/g of HCG three times a week interval at 28 – 30 ℃ are required for the female at stage one maturity, whereas a single injection is sufficient for female at stage four maturity to induce ovulation (Tan & Lam, 1973; Senoo et al., 1994a). Half of the hormone dosage received by a female is also given to the male to improve semen volume and sperm quality (Tan & Lam, 1973; Kohler et al., 1994). Besides HCG, 4 mg/kg of carp PG can also be applied to induce female ovulation (Cheah et al., 1991).

2.1.2.1.1.1 Natural spawning

Egg production via natural spawning is much easier than artificial spawning as the latter requires high skills to obtain and fertilize the eggs. Further, the former results in higher egg fertilization and hatching rates (Cheah et al., 1994; Senoo et al., 1994a). In natural spawning, hormone-treated brood fish are allowed to spawn naturally in the tank (Tavarutmaneegul & Lin, 1988; Senoo et al., 1993b; Senoo et al., 1994a). This method is similar to the spawning behaviour of fish in nature. It normally produces about 20,000 to 30,000 eggs from a 250 g female (Senoo et al., 1994a).

2.1.2.1.1.2 Artificial egg collection

The artificial spawning method is more challenging because of the difficulty of obtaining sufficient male milt, and the eggs become sticky and clump together once in contact with water (Senoo et al., 1992). Milt and eggs of marble goby are obtained using collectors which comprise of a cylindrical plastic container and two different lengths of plastic tubes connected to the container cover. The longer tube is used to suck out the eggs or milt, while the shorter tube is connected to the fish papilla. Vaseline is applied on the shorter tube and inner surface of the egg collector so as to prevent the eggs from

sticking onto the collector wall (Senoo et al., 1992). The maturity stage of a female is monitored frequently to avoid overripe eggs as it could result in low survival and hatching rate. The first success was reported using 'wet-method' by fertilizing the eggs in the petri-dishes and the eggs were collected from a 230 g brood fish. High hatching rate (90%) was recorded from 3500 eggs collected. However, the larvae survived not more than eight day after hatching (DAH) (Tan & Lam, 1973).

2.1.3 Egg development

The newly ovulated artificial fertilized eggs are transparent, ovoid and yellow-orange with long and short axes of 0.94 ± 0.09 mm and 0.62 ± 0.05 mm, respectively (Senoo et al., 1994a). 1,669.6 eggs had a total weight of 1 g. A bundle of adhesive filament develops at the basal end of the egg membrane to attach onto the substrate once the fertilized eggs are in contact with water (Tan & Lam, 1973). Interestingly, "agrippa egg" is observed where the embryonic head is located at the basal part of the egg and this is normal for marble goby but not to other gobies (Takita, 1975).

The eggs start to absorb water once fertilized leading to the elongation of eggs membrane. The size of the egg increases approximately (ca.) 53% within 10 to 15 minutes (min) (Tan & Lam, 1973; Senoo et al., 1994c) and has a diameter of 2.25 ± 0.10 mm and 0.63 ± 0.01 mm in their long and short axes, respectively (Senoo et al., 1994a). Their long axes, however, start to decrease, while their short axes begin to increase at 48 hours after fertilization (HAF). It was observed that marble goby eggs took a longer period to hatch out (38 HAF at 27 ℃ or 41 - 150 HAF at 27.2 - 27.5 ℃) than other freshwater species such as Patin (*Pangasius* spp.) and African catfish (*Clarias* spp.) at 24 HAF (Tan & Lam, 1973; Senoo et al., 1994c). The peak hatching occurs at 60 – 70 HAF when slightly pigmented embryonic eyes are formed although the hatching starts as early as 48 HAF and last until 120 HAF (Senoo et al., 1994b;

1994c). Larvae that hatched out before the peak (within 38 – 48 HAF) will die because of less development, while larvae that hatch after the peak (after 120 HAF) show deformation due to limited space in the egg shell as the larvae grow bigger (Tan & Lam, 1973; Senoo et al., 1994c).

The first cleavage happens at about 1 HAF after completion of water absorption, followed by the morula, blastula and gastrula stages which happen at 2.5, 3.5 and 5 to 7 HAF, respectively. The brain is developed at 16 HAF, while the heart pulsation starts at 29 HAF. The larvae release themselves by breaking the egg membrane using their tail, while larvae that not able to totally free themselves from the egg shell will die within one to two hours (h) (Tan & Lam, 1973; Senoo et al., 1994c).

2.1.4 Larval rearing

Proper larval feeding is crucial for the survival and growth of marble goby. The larval yolk sac is completely absorbed at 3 to 4 DAH (Widanarni, 1990; Wahyuningrum, 1991; Nasir, 1994). First feeding was observed at an average body length of 4 mm with the mouth size gape between 0.08 to 0.20 mm (Tavarutmaneegul & Lin, 1988). Basically, marble goby larvae are given various live organisms such as phytoplankton, rotifers, copepod, *Artemia* nauplii, *Moina* sp., and *chironomid* larvae according to their age. As the larvae grow bigger, larger live organisms are provided. For example, the 3 - 20 DAH larvae (0.31 - 0.41 cm) fed on only rotifers, the 21 –29 DAH larvae (0.44 - 0.65 cm) fed on both rotifers and *Artemia* nauplii and the 30 – 45 DAH larvae (0.69 - 2.15 cm) fed on only *Moina* sp. (Amornsakun et al., 2001). The length of upper jaw of larvae aged 3, 10, 20 and 30 DAH are ca. 0.20, 0.40, 0.57 and 0.80 mm, respectively (Senoo et al., 1994b). Further, the larvae are provided with 20 watts fluorescent light daily until the larvae reach about 28 DAH as the larvae are phototactic negative after that period (Senoo et al., 1994a). For instance, larvae fed with *Paramecium* sp.,

Coelestrum and *Brachionus* sp. under photoperiod of 18 h light and 6 h dark showed greater survival (11.1%) than those under dark and light of 12 h, respectively (8.0%) (Taufik et al., 2002). Further, the larvae showed higher survival and growth rate in temperature of 28 to 30 ℃ than those below 28 ℃ (Widiyati & Djajasewaka, 1992).

2.1.5 Problems in rearing larval marble goby

To date, the larval survival and growth of marble goby are still low and slow despite success in achieving high fertilization and hatching rates (Tavarutmaneegul & Lin, 1988; Senoo et al., 1993a; 1994a; 1994b; 1994c). For instances, only 147,300 juveniles were produced from 25,000,000 fertilized eggs collected from a pond (Tavarutmaneegul & Lin, 1988) and 10.1% of 16,000 hatched larvae survived up to 70 days (Senoo et al., 1994a). As a result, the larval production via hatchery remains low although the fish has been extensively reared in Thailand using various cage systems (Suwansart, 1979; Supamataya, 1984) and still heavily depended on wild caught fingerlings for stocking (Mohsin & Ambak, 1983; Ikenoue, 1991; Amornsakun et al., 2002). Eventually, the natural supply of larvae has decreased dramatically due to overfishing.

Further, most newly-hatched larvae (> 80%) did not survive past the post-larval stage and high mortality occurred especially when they switched to exogenous feeding (Tavarutmaneegul & Lin, 1988). Commonly, the newly-hatched larvae die due to starvation because of inactive feeding behaviour and unsuitable feed in the aspect of prey size or feed quality (Senoo et al., 1994b). Most of the previous studies on larval rearing were based on zooplankton fed with microalgae but this type of feed quality was unable to improve the survival and growth of marble goby larvae. Thus, it is important to find another alternative source of food to feed the zooplankton. In the present study, zooplankton (rotifers and *Artemia* nauplii) was fed with PB prior to larval feeding.

2.2 Zooplankton

2.2.1 Marine rotifer: *Brachionus* sp.

Rotifers belong to Phylum Rotifera, Class Monogononta, Order Ploimida and Family Brachionidae. These microscopic animals are filter feeding metazoans which comprise of a fixed number of 1,000 cells and grow by enlarging their plasma. They filter small particles using a ciliated corona located on the anterior portion of the body. This corona is also meant for locomotion although they spend most of their life time attach to a substrate using their retractile foot (Wendy & Kevan, 1991). Further, their epidermis consists of lorica, a densely packed layer of keratin-like proteins and their body is distinguished as head, trunk and foot (Alessandro et al., 1999).

There are two groups of marine *Brachionus* species namely, *Brachionus plicatilis*, the large (L) type and *Brachionus rotundiformis*, the small (S) type. The lorica length of L-type ranges from 130 to 340 µm (average 239 µm), whereas the S-type ranges from 100 to 210 µm (average 160 µm). These two types also differ in their weight, optimal growth temperatures and occipital spines shape (Alessandro et al., 1999).

The reproductive mode of rotifers is neither sexual (mictic reproduction) nor asexual (amictic or parthenogenetic reproduction) and depends on the environmental conditions. The clones produce asexually are genetically identical to their mothers and are diploid females. Sudden change in rotifer cultures such as changes in salinity, feed type and amount or high population densities could trigger sexual reproduction leading to produce large amounts of mictic resting eggs (Snell & Boyer, 1988; Wendy & Kevan, 1991). Amictic reproduction, however, is preferable due to faster reproductive rate and able to produce large numbers of female rotifers instead of male rotifers which are nutritionally deficient due to the absence of a functional digestive tract (Meragelman et

al., 1985). Further, an amictic female is able to produce about twenty amictic eggs or ten generations of offspring during her lifetime of seven to ten days depending on the culture temperature and the eggs are attached to the posterior portion of her body (Hoff & Snell, 1989).

2.2.1.1 Optimal rotifer culture conditions

2.2.1.1.1 pH

Rotifers are able to tolerate an environmental pH that ranges from 5 to 9 although they naturally live in environment of above pH 6.6 and its best yield is observed above pH 7.5 (Lavens & Sorgeloos, 1996). The pH concentration is correlated to the toxicity of excretion products such as unionized ammonia (NH_3) (Alessandro et al., 1999).

2.2.1.1.2 Temperature

Temperature influences the water oxygen concentration and the tolerance limits for each species depends on their physiology (Alzieu, 1990). The optimum rotifer culture temperature is strain dependent where each strain has their range of temperature requirements. For example, *Brachionus rotundiformis* grows best at temperature greater than 25 ℃, whereas *Brachionus plicatilis* exhibits better growth at below 20 ℃. The reproduction of *Brachionus rotundiformis* stops below 15 ℃, while *Brachionus plicatilis* is still productive at this temperature (Lubzens et al., 1985b). Further, L-type rotifers produce more resting eggs at 23.1 ℃, while the S-type has more resting eggs at a temperature ranges from 28.2 to 30.6 ℃ (Lubzens et al., 1985b).

2.2.1.1.3 Dissolved oxygen (DO)

Oxygen is important in rearing culture as it helps to break down the organic detritus, enables the biochemical pathways completion and for respiration. Most rotifers can withstand DO as low as 2 mg/L in water and some even survive nearly anaerobic environment for short periods (Lavens & Sorgeloos, 1996). The solubility of oxygen in culture water is correlated to the salinity, feed type, rotifer density and temperature. Temperature increases as DO decreases in culture water, while increase of rotifer metabolic rate is due to the increase of demand for DO at high temperature (Yoshimura et al., 1996).

2.2.1.1.4 Turbulence

A moderate to strong turbulence is needed to keep the food particles and rotifers in suspension. Stirring and re-suspension of bottom sediments should be kept minimal by providing a good water circulation (Alessandro et al., 1999).

2.2.1.1.5 Ammonia

The level of NH_3 is somehow related to temperature, pH and ammonium (NH_4^+). The rotifers can grow well even in high ammonia concentration (Coves et al., 1990). Hence, the toxicity of ammonia to rotifers is still unclear.

2.2.1.2 Rotifer culture systems

There are four alternative ways to culture rotifers namely, a) batch culture; b) semi-continuous culture; c) continuous culture; and d) ultra-high density culture.

2.2.1.2.1 Batch culture

In the batch culture system, the initial rotifer stocking density could range from 50 to 200 individuals/mL (ind/mL) and the culture is harvested when it reaches a density of about 600 ind/mL in ca. 4 days of cultivation (Lavens & Sorgeloos, 1996). In this system, the rotifers are totally harvested where a part of the harvested rotifers are meant for larval feeding, while the other portion is used as a starter (minimum density of 250 ind/mL) for a new culture (Agh & Sorgeloos, 2005). Basically, one of the following two strategies is applied in this system namely, a) sustaining a stable rotifer density by adjusting the culture volume; or b) increasing the rotifer density by sustaining the culture volume (Hirata, 1980; Lubzens, 1987).

2.2.1.2.2 Semi-continuous culture/thinning culture method

In the semi-continuous culture system, the density of rotifers is maintained constantly by harvesting ca. 25% of culture volume daily and the same amount of new water is replaced back to the culture (Girin & Devauchele, 1974). This system is basically applied in a large container of about 50 – 200 m^3 and the rotifer density of 300 to above 1000 ind/mL can be achieved in 3 to 7 days of cultivation from the initial stocking density of 50 to 200 ind/mL.

2.2.1.2.3 Continuous culture

The function of this culture system is similar to that in semi-continuous culture system so as to maintain an excellent water quality in order to sustain a high quality and constant rotifer density. Although it provides higher productivity, compared to batch and semi-continuous culture systems, it is costly and its operating system is complicated.

2.2.1.2.4 Ultra-high density culture

This culture system is equipped with a unit of porous membrane filtration for continuous renewal of culture water containing high ammonia concentration with fresh seawater in order to increase the rotifer productivity and vitality (Yoshimura et al., 2003). The advantages of this culture system are that it does not require a large culture area and only minimal labour is required. A density of 20,000 to 40,000 ind/mL can be achieved using this culture system (Fu et al., 1997).

2.2.1.3 Rotifer diets

In nature, rotifers fed on variety of microorganisms such as microalgae, yeast, protozoa and bacteria (Fukusho, 1989). Microalgae such as *Nannochloropsis oculata*, *Tetraselmis tetrahele* and *Isochrysis galbana* are the most common rotifer diets. *Nannochloropsis oculata* with a size of 1 to 2 μm in diameter is rich in eicosapentaenoic acid (EPA), while *Isochrysis galbana* contains a high amount of docosahaxaenoic acid (DHA). Besides being used as feed, microalgae can also act as bacteriostatic and water conditioner in rotifer cultures. Nonetheless, the cultivation of microalgae requires more time, labour and facilities. Further, the nutritional content and punctuality of microalgae are questionable under large production conditions (Fukusho, 1983). On the other hand, yeasts such as baker's yeast (*Saccharomyces cerevisiae*), marine yeast (*Torulopsis candida var. marina*, *T. larvae*, *Saccharomyces acidosaccharophill* and *Zygosaccharomyces marina*) and caked yeast (*Rhodotorula)* are commonly used to feed rotifers. Baker's yeast with the size of 5 to 7 μm in diameter is protein-rich and usually use as a microalgal substitute for rotifer (Hirayama, 1987). However, yeast lacks several essential fatty acids (EFA) and vitamin B_{12} which are essential for rotifer reproduction. Thus, finding a new source of food for enriching and enhancing rotifer production is needed. In this study, rotifers are fed with PB grown in POME.

2.2.1.4 Biochemical content of rotifers

The rotifers can be short-term (ST) and/or long-term (LT) enriched by manipulating their biochemical compositions such as lipids, n-3 HUFA, n-6 fatty acid and protein contents as the nutritional value of rotifers is positively correlated to its diet. ST-enrichment is done within one generation time or less than 24 h, whereas LT-enrichment is carried out for few generation times or more than 24 h prior to harvesting them for larval feeding (Planas & Cunha, 1999; Dhert et al., 2001). Generally, LT-enrichment is for both growth and usually lipid enhancement during the rearing of rotifers (Planas & Cunha, 1999). Both methods have advantages and disadvantages. For instance, although ST-enrichment is flexible and fast, it often leads to bad hygienic quality and poor quality rotifers with an extremely high lipid content (Dhert et al., 1990; Støttrup & Attramadal, 1992). Further, most uneaten ST-enrichment rotifers will lose their nutritive value if not eaten immediately by larval fish. On the other hand, although LT-enrichment gives better and more stable nutritional quality of rotifers, the increased growth rate of LT-rotifers eventually results in the decrease of n-3 HUFA content and DHA/EPA ratio and the increase of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) contents in rotifers (Planas & Cunha, 1999). Further, the rotifers tend to utilize DHA at high growth rate leading to higher EPA than DHA in their tissue (Planas & Cunha, 1999). However, the incorporation of nutrients in rotifer is easier when compared to *Artemia* in that rotifer does not catabolize HUFA (Bell et al., 1995), whereas *Artemia* does (Dhert et al., 1993) and often catabolise DHA back to EPA (Cortney et al., 2009).

Little attention has been paid to protein compared to lipids in rotifers. Basically, the protein content of rotifers is varied among different operational culture conditions and feeding. However, the variations among the amino acid and protein contents in rotifers fed with different diets are relatively low (Watanabe et al., 1983a; Lubzens et al.,

1989; Øie et al., 1997). Generally, the protein profile of rotifer ranges from 28 to 67% of dry weight (DW), whereas its amino acid content is constant and negatively related to the feed quality (Lubzens et al., 1989).

2.2.2 Brine shrimp, *Artemia* sp.

Artemia belongs to Phylum Arthropoda, Class Crustacea, Order Anostraca, Family Artemidae and Genus *Artemia* (Alessandro et al., 1999). These small branchiopod crustaceans are able to survive in extremely hostile conditions and are widely distributed. They can also survive in freshwater for short periods but unable to reproduce (Granvil, 2000). They are non-selective filter feeding where they feed on microorganisms such as bacteria and algae of suitable size. They can withstand salinity of above 200 ppt which give them an advantage of being not preyed by predators as they are defenceless. Further, they regulate their osmotic blood values by absorbing water from the medium and discarding the salt by defecation in order to survive in hyperhaline environment (Alessandro et al., 1999).

The most unique characteristic of *Artemia* is its capability to produce resistant cysts, comprising embryos in diapauses or dormant stage (Van Stappen, 1996). When the environmental conditions are unfavorable them, they will produce cysts to protect their embryos from extreme temperature and salinity as well as dehydration. The cysts of about 200 to 300 µm in diameter start to hatch out after 15 to 20 h in seawater. The larva will go through about fifteen molts prior to reaching adulthood in about eight days with 400 to 500 µm of initial size. The male is distinguished from female by their pair of huge muscular claspers (the second pair of antennae) in the head region. Further, the female carries the brood pouch or uterus behind the eleventh pair of thoracopods (Alessandro et al., 1999).

Artemia can perform parthenogenetic and bisexual reproduction where ovoviviparity and oviparity prevail. Change of reproduction mode from ovoviviparous to oviparous happens in under nourishment or insufficient feed quality rather than influences by abiotic conditions (Alessandro et al., 1999).

2.2.2.1 Cultivation of *Artemia* sp.

High hatching rate of *Artemia* cysts can be achieved by providing the following optimal conditions, a) salinity of 5 ppt; b) temperature of 28 ℃; c) strong and continuous aeration supply of about 4 g/L; d) a pH of about 8; e) stocking density of not more than 5 g of cysts in a litre of water; and f) constant illumination of 2 Klux (Granvil, 2000). It is recommended to hatch the *Artemia* cysts in translucent cylindrical tanks with conical bottoms for ease in harvesting and separating the hatched nauplii from unhatched cysts and empty shells. Sometimes, the shell of *Artemia* cysts is removed or decapsulated prior to incubation. This process provides several advantages such as, a) able to produce more digestible nauplii even unhatched; b) shorten the hatching period; c) nauplii much easier to emerge; d) better quality of newly-hatched nauplii; and e) provides smaller size of decapsulated cysts for larvae with small mouth gape (Granvil, 2000). The decapsulation of *Artemia* cysts involves three main steps namely, a) re-hydration; b) decapsulation using chlorine; and c) washing and deactivation of the residual chlorine (Granvil, 2000). The instar I *Artemia* nauplii are a non-feeder and do not require feeding but their later life stages must be fed prior to larval fish feeding due to the low levels of n-3 HUFA, particularly DHA in their body. The success in manipulating their nutritional contents is dependent on the strain itself, the culture conditions during enrichment and quality of the diet (Han et al., 2000). *Artemia* nauplii are commonly fed with algae (Watanabe et al., 1983a), ω-yeast (Watanabe et al., 1983b), microparticles (Léger et al., 1985), liposomes (Ozkizilick & Chu, 1994),

microencapsulated diets (Southgate & Lou, 1995) and emulsion (Léger et al., 1987, McEvoy et al., 1996).

2.2.2.2 Biochemical composition of *Artemia* sp.

Generally, the *Artemia* cysts comprise of 10% fat, 10% crude fiber and 28% crude protein, whereas the newly-hatched *Artemia* nauplii contain 13 to 19% fat, 3 to 15% n-3 HUFA, 14 to 15% carbohydrate and 51 to 55% protein (Granvil, 2000). However, the lipid and fatty acid profiles of *Artemia* nauplii are significantly varied among strains and year class with the freshly hatched *Artemia* nauplii being the most nutritious (Granvil, 2000). In contrast, their amino acid compositions are relatively similar among different strains, indicating that it is not geographically determined unlike their fatty acid content (Agh & Sorgeloos, 2005). Further, *Artemia* can be categorized into two types based on their fatty acid composition namely, a) the freshwater type, which contains a high content of LNA; and b) the marine type, which contains a high amount of EPA (Watanabe et al., 1978).

2.2.3 Advantages of *Brachionus* sp. and *Artemia* sp.

Rotifers and *Artemia* are the most common live food use for larval feeding. Larval fish that have small mouth gape must be initially fed with rotifers prior to switching to *Artemia*. The advantages of rotifers are due to their characteristics of being small size, high reproductive rate, able to survive in a wide range of salinities, easy to enrich with exogenous nutrients, slow swimming behaviour, habit of suspending in the water column and capable of being cultured at high densities (Hirata, 1979). On the other hand, the advantages of *Artemia* include easy to produce, are visible as prey and availability of dormant cysts (Alessandro et al., 1999). Nevertheless, the major constraint about these two zooplanktons as live organisms for larval fish is their

nutritional quality where they lack several EFA such as DHA, EPA or ARA (Table 2.1). In this study, these zooplanktons were fed with PB prior to larval feeding as PB are rich in nutrients.

Table 2.1 The composition of highly unsaturated fatty acids (HUFA) (weight % total fatty acids) in total lipid of unenriched rotifers and *Artemia* nauplii

Sample/ HUFA	DHA	EPA	ARA	DHA/EPA	EPA/ARA
Unenriched rotifers	0.1	0.2	Trace	0.5	-
Unenriched *Artemia* nauplii	0.0	5.3	1.2	0.0	4.1

Adapted from Bell et al., 2003

2.3 Phototrophic bacteria

2.3.1 Definition

Phototrophic bacteria previously known as photosynthetic bacteria are prokaryotes that use light energy to metabolise useful chemical energy via either chlorophyll- or bacteriochlorophyll-mediated processes (Imhoff, 1992). They derive energy from light by photophosphorylation to synthesis organic materials from inorganic components during photosynthesis (Imhoff, 1992).

2.3.2 Classification and morphology

Phototrophic bacteria are widely distributed in nature such as in aquatic, terrestrial and even in extreme environments such as in the Antarctica (Herbert, 1976; Madigan, 1999). They undergo photosynthesis and consist of light-harvesting 1 (LH-1), light-harvesting 2 (LH-2) and a photosynthetic apparatus also known as the reactor center (RC) (Masuda et al., 2000). The light energy absorbed by LH-1 and LH-2 is transferred to the RC by bacteriochlorophylls to activate the primary photochemical reaction (Imhoff, 1995). They utilize various sources of carbon such as organic acids, alcohol, aromatic compounds and carbohydrates and require growth factors such as vitamins and complex organic nitrogen sources (Getha, 1995; Imhoff, 1995).

There are two groups of PB, namely oxygenic PB (cyanobacteria) and anoxygenic PB. These two groups are distinguished by their photosynthetic pigments, structure and complexity of the photosynthetic apparatus (Stainer et al., 1981). The cyanobacteria are aerobic bacteria with two photosystems which use carbon dioxide and light energy to obtain chemical energy and oxygen during photosynthesis, whereas the anoxygenic PB have only one photosystem to evolve oxygen as a final product but unable to use water as an electron donor. Unlike cyanobacteria, anoxygenic PB are able to utilize various simple organic compounds as electron donors in the presence of light (Pfennig, 1977; Imhoff, 1992).

2.3.3 Anoxygenic phototrophic bacteria

There are two major groups of anoxygenic PB, namely green and purple bacteria. The green bacteria are represented by the green and brown sulfur bacteria (Family Chlorobiaceae) and the filamentous gliding bacteria (Family Chloroflexaceae), while the purple bacteria are represented by the purple sulfur bacteria (Family Chromatiaceae and Ecthiorhodaceae) and the purple non-sulfur bacteria (PNSB) (Family Rhodospirillaceae). The green and brown sulfur bacteria are strictly anaerobic and light dependent, while the filamentous gliding bacteria are mainly facultative aerobic and photo-organotrophic. Unlike green bacteria, the purple sulfur bacteria assimilate carbon dioxide anaerobically in the presence of reduced sulfur and accumulate elemental sulfur, whereas the PNSB are photoheterotrophic which use simple organic substrates as both carbon source and electron donors under anaerobic-light condition but do not utilize elemental sulfur (Smith, 1988). The green and purple bacteria are further distinguished from each other based on their membrane structure and their composition of lipids, fatty acids and quinines (Imhoff, 1992).

The anoxygenic PB are in oxygen-deficient conditions during photosynthesis due to the repression of photosynthetic pigments by oxygen and prefer light at longer wavelengths to yield less energy for photosynthesis. They require electron donors of lower redox potential than water such as sulfide, other reduced sulfur compounds, organic molecules and molecular hydrogen (Sasikala et al., 1993; Imhoff, 1995). Besides, they can also utilize reduce iron as electron donor (Widdel et al., 1993).

2.3.4 Purple non-sulfur bacteria (Athiorhodaceae)

The PNSB have been intensively utilized because of their characteristics, namely able to grow in various substrates and culture conditions, easy to enrich and abundant in nature (Imhoff & Truper, 1989). They are named "non-sulfur" as they were originally believed to be unable to utilize hydrogen sulfide as an electron donor to reduce carbon dioxide to cell material (Madigan et al., 2000). Eventually, it was reported that these species are able to utilize sulfide at levels that are non-toxic to them (Madigan et al., 2000). They are also Gram negative, rod-shaped to ovoid and multiple by budding. Most species are motile by flagella, need growth factors and do not formed gas vesicles (Imhoff, 1992). They differed from other bacteria by their internal membrane structure, morphology, composition of carotenoid, utilization of electron donors and carbon sources. They have only one photosystem which comprises of three components, namely an antenna of light to harvest pigments, an electron transfer chain and a centre of photochemical reaction (Kondratieva & Krasil'nikova, 1981).

Purple non-sulfur bacteria have a wide range of growth modes and able to grow under both anaerobic-light and aerobic-dark conditions (Sasikala et al., 1993). For instance, *Rhodopseudomonas sphaeroides* (Nishizawa et al., 1974) and *Rhodopseudomonas capsulate* (Madigan et al., 1980) are able to grow in both conditions. They can grow photoheterotrophically under anaerobic-light condition using

various organic substrates as carbon sources and electron donors (Van Niel, 1957). Normally, they use C5 – C18 straight-chain SFA as their carbon source. Some species even grow well when given organic carbon sources such as amino acids, organic acids, carbohydrates, aromatic organic compounds and alcohols (Imhoff & Truper, 1992). They are also able to grow photoautotrophically with hydrogen or sulfide as electron donors and carbon dioxide as carbon source (Hallenbeck et al., 1990). Most species oxidize sulfide to elemental sulfur but some species such as *Rhodopseudomonas palustris* form sulfate without accumulating the elemental sulfur which is an intermediate product generated during oxidation to sulfate (Hansen, 1974).

Moreover, PNSB can also be cultured under microaerobic to aerobic conditions in the dark as chemoheterotrophs or as chemolithotrophs (Imhoff, 1995). They grow chemolithotrophically in the dark using carbon dioxide, oxygen and hydrogen as their energy source, carbon source and terminal electron acceptor, respectively (Madigan & Gest, 1979). For example, *Rhodobacter sphaeroides* grew well under microaerobic-light, anaerobic-light or aerobic-dark condition (Sasaki et al., 1991). Nonetheless, the growth rate of bacteria and chemical oxygen demand (COD) reduction in aerobic-dark and microaerobic-light cultures are different from anaerobic-light culture (Sasaki et al., 1991). Further, most species of this group are not sensitive to oxygen and eventually grow well under aerobic-dark condition but have colourless cultures due to suppression of photosynthetic pigment production by oxygen. Only a small number of PNSB are unable to tolerate the presence of oxygen (Imhoff, 1995). The aerobic PB use organic substrates as their main energy and carbon sources for biosynthesis (Yurkov & Beatty, 1998). In contrast, the growth of anaerobic PB is supported by respiratory electron transport in the presence of nitrite, nitrate, dimethylsulfoxide (DMSO), nitrous oxide or trimethylamine-N-oxide (TMAO) as electron acceptors under dark condition (Imhoff & Truper, 1992).

2.3.5 Application of phototrophic bacteria

The use of PB appears limitless and has been applied in various areas including environmental study, agriculture industry and other fields that directly or indirectly use these bacteria.

2.3.5.1 Environmental biotechnology

2.3.5.1.1 Bioremediation of agro-industrial wastewater

Agro-industrial effluents constitute a very serious issue of environment pollution. For example, the palm oil industry discharges ca. 4.5 million metric tonnes of untreated effluents into the waterways every year (Noor Hazira, 2006) and this amount will increase in the years to come as the industry keeps expanding. Thus, proper waste treatment should be done before discharge. PB are well known for their ability to bioremediate high organic concentrations of wastewater (Kobayashi & Kobayashi, 2001). Indeed, they have been widely applied in conventional wastewater treatment processes. For instance, PB have been used in treating rubber sheet effluent in Thailand, successfully reducing the BOD (biochemical oxygen demand) by 70% and COD by 54% (Kantachote et al., 2005). They have also been used to treat cow dung and swine wastes (Sasaki et al., 1991). Further, organic molecules found in agro-industrial or food wastes serve as a growth medium for PB (Kobayashi & Kobayashi, 1995). This enhances bacterial growth and indirectly increases the process of bioremediation. For example, *Rhodovulum sulfidophilum* grown in sardine processing wastewater successfully reduced the COD of sardine processing wastewater by up to 85% after 120 h of cultivation (Azad et al., 2003). In addition, there are several advantages of using PB in treating wastewater namely, a) no dilution of wastewater is required; b) little space is required for treatment facilities (Sasikala & Ramana, 1995; Kobayashi & Kobayashi,

2001); and c) the waste-grown bacterial biomass is rich in nutrients with potential use as an aquaculture feed (Azad et al., 2002).

2.3.5.2 Aquaculture biotechnology

Phototrophic bacteria have been intensively used in aquaculture industry mainly because of three reasons: They are a) able to bioremediate various kinds of wastes (Kobayashi & Kobayashi, 1995); b) a source of antibiotics in feed additives due to probiont properties in bacterial cells (Banerjee et al., 1999); and c) highly nutritious (Kobayashi & Kobayashi, 2001). They can be used directly to feed the fish and prawn larvae or indirectly via a zooplankton such as rotifers or *Artemia* sp. For example, the supplement of 1% of fresh *Rhodovulum sulfidophilum* biomass to the standard *Skeletonema costatum* feed gave higher larval prawn survival (27%) and mean total length (6.13 mm) than those fed with standard *Skeletonema costatum* alone at the end of first post-larval stage (PL1) (Azad, 2004). PB could also prevent the prawn from gill disease which had caused great economic loss in Japan (Okamoto et al., 1988). Further, larval *Oreochromis niloticus* fed with commercial fish feed supplemented with *R. sulfidophilum* showed 16% higher survival than those fed with only fish feed (Banerjee et al., 1999).

2.3.6 Why phototrophic bacteria?

Phototrophic bacteria are highly nutritious and very versatile. Cultivation of PB in municipal and agro-industrial wastes is not only treating the effluents but its biomass could be also a potent single cell protein (SCP) source for animal feedstocks as well as feed supplements for aquaculture products.

2.3.6.1 High nutritive values

The nutritional quality of PB is influenced by culture conditions, substrates and strains used. The PB are rich in various vitamins especially vitamin B_{12} (30 - 79 mg vitamin B_{12}/kg dry cell weight) which is very important in the medicinal field and aquaculture industry as an animal feed supplement (Sasaki et al., 1991). The biomass also has high amounts of active compounds and enzymes. Besides, they also contain significantly large numbers of biological co-factors and carotenoid pigments (0.09 - 0.80 mg carotenoid/g dry cell). Carotenoids in PB not only protect the bacterial cells from harmful radiation but are also important in light-harvesting during photosynthesis (Ratledge & Wilkinson, 1989). These carotenoids tend to increase the viability and survival of teleost eggs as well as serve as colour-intensifying substances for ornamental fish and egg yolk (Noparatnaroporn et al., 1987).

2.3.6.2 Single cell protein

Single cell protein is defined as dried and dead microorganism cells grown in various carbon sources for their protein content (Krishna & Young, 2004). These microorganisms include yeast, algae, fungi and bacteria. The SCP is meant as feed supplement for livestock and human consumption (Scragg, 1999). PB are preferable than algae and yeast as SCP because of their high nutritional profile (Kobayashi & Kobayashi, 1995). The protein content of bacterial cells (60 - 70%) is superior to that of yeasts (40 - 60%) (Sasaki et al., 1998) and is comparable to algae (50 - 60%), egg and soybean meal (Kobayashi & Kurata, 1978, Noparatnaraporn et al., 1987) (Table 2.2). Further, PB have a short generation time.

Table 2.2 The composition of phototrophic bacteria (PB), algae and yeast (g/100g dry weight) (Kobayashi & Kobayashi, 1995)

Proximate composition	PB	Algae	Yeast
Crude protein	60.95	55.52	50.50
Crude lipid	9.91	8.07	1.1
Crude fibre	2.92	12.09	2.1
Soluble carbohydrate	20.83	21.04	39.3
Ash	5.39	3.28	7.0

The cultivation of PB in agro-industrial waste to produce SCP dates as far back as the 1960s. For instance, *Rhodocyclus gelatinosus* was grown in soybean waste and cassava starch for their bacterial cells as SCP and to reduce the COD concentration in the waste (Sasaki et al., 1981; Noparatnaraporn et al, 1987). The production of SCP from natural resources is safer for human and animal consumption and shows high nutritional profile due to the availability of variable organic compounds. The nutritional profile of PB can be modified by genetic manipulation and they can be grown in widely available raw carbon substrates or agro-industrial effluents in a limited space without affecting their production. Further, the benefits of waste-grown PB as animal feed supplement includes, a) rich in protein, amino acids and vitamins; b) soft and digestible cell wall; c) non-toxic and non-pathogenic; d) cheap biomass; and e) transportable as dried cells (Kobayashi & Kurata, 1978; Noparatnaraporn & Nagai, 1986).

2.3.7 Limitation of phototrophic bacteria

The PB have a limitation although their nutritive value is comparable with other conventional feeds as reported by Shipman et al. (1977), Kobayashi & Kobayashi (1995) and Getha et al. (1998a; b). Most PNSB have similar fatty acids profiles and basically lack polyunsaturated fatty acids (PUFA) although they are rich in C-16 and C-17 SFA and MUFA (Imhoff & Imhoff, 1995). The bacterial polar lipids and fatty acids compositions are influenced by substrate types and growth environments such as limited phosphate which increased the proportion of sulfolipid in *Rhodobacter sphaeroides*

(Imhoff & Imhoff, 1995). Temperature also influences bacterial fatty acids composition. High temperature increases C-16 and SFA, but decreases C-18 and unsaturated fatty acids in bacteria due to the regulation of fluidity and bi-layer stability of the membrane (Imhoff & Imhoff, 1995). To address this problem, the PB should be grown in substrate which has adequate EFA especially DHA, EPA and ARA. In this study, the PB were grown in POME, a by-product of palm oil production which is organic and non-toxic. Further, it has oils that may be utilized by PB during growth.

2.4 Palm oil industry in Malaysia

2.4.1 Palm oil production

Oil palm (*Elaeis guineensis*), a native of West Africa was first introduced into Malaysia in 1870 (MPOC, 2006) but the development of the oil palm industry was slow (Imam, 1984). It grew dramatically after synthetic rubber was successfully produced and subsequently threatened the natural rubber industry (Polhamus, 1962). The rubber plantations started to gradually switch to oil palm cultivation due to the high demand for palm oil, palm kernel cake and palm kernel oil (Hartley, 1967) and low demand for natural rubber in the 80s. Eventually oil palm replaced rubber as the main plantation crop in Malaysia. Today, Malaysia is one of the largest producer and exporter of crude palm oil in the world. The production of crude palm oil of only 1.3 million tonnes in 1975 had increased dramatically to 16.99 million tonnes in 2010 (Malaysian Palm Oil Board – MPOB, 2010). Further, it is the highest yielding oil crop with 4 to 5 tonnes of oil/ha/year and is about ten times soybean's yield. It is forecasted that the demand of palm oil will be greater in the years to come as the world demand for total fats and oil continues to increase.

2.4.2 Palm oil mill effluent

Palm oil mill effluent is one of the most polluting agro-industrial wastes in Malaysia. It is produced during the crude oil extraction process and is derived from the sterilizer condensate (ca. 36% of total POME), separator sludge (ca. 60% of total POME) and hydrocyclone waste (ca. 4% of total POME) (Industrial Processes and the Environment, 1999). The characteristics of each individual wastewater streams generated from the three main sources and their combination are shown in Table 2.3. Further, 1.5 cubic meters of water is needed to process one tonne of fresh fruit bunch and 50% of water will end up as POME and the other 50% being lost as steam (Ahmad et al., 2003).

Table 2.3 The characteristics of individual waste streams and combined POME

Parameters*	Sterilizer condensate[a]	Separator sludge[a]	Hydrocyclone waste[a]	Combined POME[a]		Combined POME[b]	Combined POME[c]	Combined POME[d]
				Mean	Range			
pH	5.0	4.5	-	4.2	3.4 - 5.2	4.15 - 4.45	3.5 - 4.7	4.33 ±0.3
Oil and grease	4,000	7,000	300	6,000	150 - 18,000	1,077 - 7,582	4,000	2,151.0 ± 50.1
BOD; 3-day, 30 ℃	23,000	29,000	5,000	25,000	10,000 - 44,000	21,500 - 28,500	10,250 - 43,750 (BOD_5)	35,580.0
COD	47,000	64,000	15,000	50,000	16,000 - 100,000	45,500 - 65,000	16,000 - 100,000	113,191.0
Total suspended solid	5,000	23,000	7,000	40,500	11,500 - 79,000	15,660 - 23,560	410 - 60,360	18,980.0
Total dissolved solids	34,000	22,000	100	18,000	5,000 - 54,000	15,500 - 29,000	NA	NA
Ammoniacal-nitrogen	20	40	-	35	4 - 80	NA	35	NA
Total nitrogen	600	1,200	100	750	80 - 1,400	500 - 800	200 - 500	NA
Carbon (%)								36.36 ± 3.8
Nitrogen (%)								2.71 ±0.9
C/N								13.4

*All units are in mg/L exclude pH, carbon, nitrogen and C/N; NA – Not Available; [a] Industrial Processes and the Environment, 1999; [b] Wong et al., 2009; [c] Foo & Hameed, 2010; [d] Azhari et al., 2010

Palm oil mill effluent is a thick brownish colloidal suspension which contains 95 to 96% water, 4 to 5% solids residue and 0.5 to 0.7% residual oil (Ma, 2000). Besides, POME is rich in organic carbon with BOD of 20,000 ppm and its BOD is hundred times greater than domestic sewage (Gurmit et al., 1999; Ma et al., 2001). Further, it also contains a very high level of surface active compounds (Chow & Ho, 2002) and appreciable amounts of phosphorus, potassium, magnesium, calcium and others (Table 2.4).

Table 2.4 Minerals composition in raw POME

Minerals	Raw (POME) (µg/g DW)[a]	Raw POME (mm/g DW)[b]	Raw POME (mg/L)[c]	Raw POME[d]
Essential minerals				
Aluminum (Al)	16.60 ±1.44	97.95 ±0.48	NA	NA
Arsenic (As)	9.09 ±0.65	0.74 ±0.02	NA	NA
Boron (B)	7.60 ±0.60	2.21 ±0.06	NA	95.59 ±8.2 mg/kg
Calcium (Ca)	1650.09 ±160.45	143.55 ±1.16	276 - 405	1.56 ±0.1%
Cobalt (Co)	2.40 ±0.35	0.05 ±0.002	0.04 - 0.06	NA
Chromium (Cr)	4.02 ±0.44	0.79 ±0.02	0.05 - 0.43	NA
Copper (Cu)	10.76 ±1.04	1.58 ±0.03	0.8 - 1.6	73.24 ±8.1 mg/kg
Iron (Fe)	11.08 ±2.20	186.61 ±1.84	75 - 164	1.03 ±0.3%
Potassium (K)	8951.55 ±256.45	39.52 ±0.45	1281 - 1928	2.49 ±0.2%
Magnesium (Mg)	911.95 ±95.50	24.35 ±0.57	254 - 344	1.21 ±0.2%
Manganese (Mn)	38.81 ±3.65	1.46 ±0.08	2.1 - 4.4	339.0 ±20.0 mg/kg
Molybdenum (Mo)	6.45 ±0.40	0.46 ±0.03	NA	NA
Sodium (Na)	94.57 ±6.45	42.39 ±0.58	NA	NA
Nickel (Ni)	1.31 ±0.30	0.24 ±0.004	NA	NA
Phosphorus (P)	14377.38 ±1206.88	646.63 ±4.19	94 - 131	1.01 ±0.2%
Sulfur (S)	13.32 ±1.45	7.02 ±0.11	NA	0.57 ±0.2%
Selenium (Se)	12.32 ±1.35	1.59 ±0.09	NA	NA
Silicon (Si)	10.50 ±1.80	15.16 ±0.29	NA	NA
Tin (Sn)	2.30 ±0.30	12.42 ±0.11	NA	NA
Vanadium (V)	0.12 ±0.02	0.32 ±0.01	NA	NA
Zinc (Zn)	17.58 ±2.10	4.72 ±0.12	1.2 - 1.8	118.82 ±22.1 mg/kg
Non-essential minerals				
Cadmium (Cd)	0.44 ±0.06	0.05 ±0.002	0.01 – 0.02	1.2 ±0.1 mg/kg
Lead (Pb)	5.15 ±0.55	3.45 ±0.11	NA	NA

NA – Not Available; [a] Habib et al., 1997; [b] Habib et al., 1998; [c] Wong et al., 2009; [d] Azhari et al., 2010

2.4.3 Applications of palm oil mill effluent

Generally, POME has been used as a fertilizer and animal feed (Ta et al., 2009). It is also used as a fermentation medium to produce a variety of metabolites or products such as bioinsecticides, antibiotics, polyhydroxyalkanoates, solvents, enzymes and organic acids (Wu et al., 2007) as well as hydrogen (Atif et al., 2005; Vijayaraghavan & Ahmad, 2006). Nevertheless, the use of POME in aquaculture sector is limited and unexplored. In the present study, POME is used as a substrate to culture PB which in turn is used as an alternative aquaculture feed for zooplankton and larval fish.

CHAPTER 3

SELECTION OF SUITABLE PHOTOTROPHIC BACTERIUM FOR AQUACULTURE

Summary of significant findings

The production of high quality live feed at the cheapest possible cost is crucial for the aquaculture industry. Little attention has been paid to the utilization of phototrophic bacteria (PB) grown in palm oil mill effluent as live microbial feed for zooplankton and larval fish cultures. Although PB grew better in synthetic 112 medium as compared to POME, it was shown that PB could grow in the oily waste. PB grown in 112 medium had significantly higher biomass, total carotenoids and daily growth rate than PB grown in POME irrespective of bacterial species used. Rotifers fed with biomass of PB grown in 112 medium had significantly higher density than those fed with biomass of PB grown in POME. However, marble goby larvae survived until the end of 30 days rearing period when given live feed (rotifers and/or *Artemia* nauplii) fed with biomass of PB grown in POME, whereas those given live feed fed with biomass of PB grown in 112 medium did not survive. Further investigations had shown that only fish larvae reared on live feed fed with biomass of *Rhodovulum sulfidophilum* (PD1) grown in POME (bPOME-PD1) survived until the end of the feeding trial, while larvae reared on live feed fed with biomass of *Rhodopseudomonas palustris* (B1) or *Rhodobacter sphaeroides* (KS) grown in POME (bPOME-B1 and bPOME-KS, respectively) did not. Only bPOME-PD1 contained both DHA and EPA. Hence, bPOME-PD1 has the potential to be used as a live microbial feed in aquaculture.

3.1 Introduction

Microorganisms and in particular, the PB have been widely utilized in the aquaculture industry as live microbial feed and their beneficial effects have been reported (Madukasi et al., 2010). The use of bacterial biomass in aquaculture diet has several advantages over conventional feed ingredients as reviewed in Section 2.3.6.2 (page 32). Further, the cost of production is low when compared to the production of other live microbial feed such as algal biomass. Their efficacy as a nutritious and non-toxic feed has been attested by the increased survival and growth of fish larvae, increased fecundity and rapid development of gonads and skin colour enhancement of shrimps and carps (Noparatnaraporn & Nagai, 1986). Some strains also produce antiviral substances against some fish viruses such as swellfish (Hirotani et al., 1991). It was reported that there is no or very reduced mortality when 0.1% (w/w) live PB is added to the formula feed given to the crucian carp fry soon after hatching (Okamoto et al., 1988; Hirotani et al., 1991). Further, larvae of *Penaeus chinensis* were successfully produced on a diet comprising four strains of PB (Cui et al., 1997). The addition of PB as feed supplement also favours the growth of zooplankton especially brine shrimp's growth and it was superior to green algae (Kobayashi, 1995).

However, many PB species lack PUFA and HUFA which are essential for the survival of larval fish. For instance, koi (*Cyprinus carpio*) larvae are unable to survive when given a sole diet of freeze dried 112-PD1 as the larvae were inactive, slow to respond and listless after 7 days of rearing (Neik, 2006). This is likely due to the use of a diet containing a low amount of total lipid (1.5 – 9.1%) (Csengeri et al., 1978). Mixed diet containing freeze-dried 112-PD1 and "Omega Plus" hen egg yolk, however, resulted in higher survival and growth of koi larvae. Since freeze-dried 112-PD1 contains relatively high amount of protein and carbohydrate (Neik, 2006), the factor responsible for larval survival is likely lipids, in particular PUFA and HUFA, present in

the egg yolk. Thus, it is hypothesized that PB grown in synthetic medium lacks essential fatty acids (EFA) or/and their precursors that are essential for survival of fish larvae. This hypothesis will be tested in this study.

Highly unsaturated fatty acids play a major role in sustaining the structure and function of cell membrane, proper development and functioning of visual and neural systems as well as stress tolerance (Kanazawa, 1997; Rainuzzo et al., 1997; Sargent et al., 1997). The deficiency of these EFA results in poor growth, susceptibility to stress and disease, anemia, low feed efficiency and high mortality. It was also reported that poor dietary EFA in fish gave rise to abnormal pigmentation, fatty liver, poor swimming activities, and raised basal cortisol levels. For instance, groupers need at least 14% (w/w) lipids to ensure good growth (Boonyaratpalin, 1997).

Although PB species lack PUFA and HUFA, the culture of PB in wastewaters rich in lipids may result in biomass containing these fatty acids. Further, PB are also very effective in reducing the pollution load of wastewaters containing high organic concentrations (Kobayashi & Kobayashi, 1995). However, the use of sewage and animal wastes as substrate for PB could give rise to contamination by pathogenic bacteria and the substrate may contain harmful substances (Shipman et al., 1977). In contrast, substrates from agro-industries are less hazardous with less microbial contamination. Thus, POME a major agro-industrial pollutant (80%) in Malaysia with BOD of 25,000 ppm was selected in this study. It was reported that for every tonne of palm oil generated about 2.5 tonnes of wastewater was produced. Although POME is considered as a pollutant, it may have applications in aquaculture industry. Several studies have shown the recovery of nutrients from POME as a potential nutrient source for fish (Phang, 1990). As POME contains 4000 - 6000 mg/L of oil and grease, the extract of the oil droplets contained 84 wt% neutral lipids and 16 wt% of complex lipids. Moreover, POME contained high concentrations of surface active compounds such as

glycolipids (6%) and phospholipids (10 wt%). Further, POME also contained high concentrations of carbohydrate, protein, lipids, minerals and nitrogenous compounds. The high nutrients of POME also support the growth of several microorganisms including PB (Zadariana et al., 2009). There are dual benefits derived during the growth of the bacteria as the organic nutrients utilized by the bacteria produced lipids-rich live microbial feed for aquaculture with simultaneous reduction of the pollution load of POME.

Hence, the main objective of this part of the study was to utilize the nutrients of POME to culture PB as a live microbial feed for aquaculture. The specific aim was to evaluate the benefits of biomass of PB grown in POME as direct feed for rotifer and indirect feed (via live feed such as rotifers and *Artemia* nauplii) for marble goby larvae.

3.2 Materials and methods

3.2.1 Bacteria cultures

The stock cultures of PNSB were obtained from Mycology and Plant Pathology Laboratory, University of Malaya. The freshwater species, *Rhodopseudomonas palustris* strain PBUM001 (B1) and *Rhodobacter sphaeroides* strain PBUM003 (KS) were isolated from starch noodles ('bihun') processing wastewater and seafood processing wastewater, respectively, while the marine species, *Rhodovulum sulfidophilum* strain PBUM002 (PD1) was isolated from mangrove mud (Figure 3.1). The master stock cultures of the PB were maintained as stab cultures on 112 medium (Gest & Favinger, 1983) solidified with 1.5% (w/v) agar. These stab cultures were incubated at 30 ± 2 ℃ for 48 h and topped with sterile paraffin oil. The cultures were then kept at 4 ± 2 ℃ (Azad et al., 2001). Prior to use, the stock cultures were plated out and checked for purity.

Figure 3.1 The three species of phototrophic bacteria (PB) used for rotifer production and larval feeding. A = *Rhodopseudomonas palustris* (B1); B = *Rhodobacter sphaeroides* (KS); C = *Rhodovulum sulfidophilum* (PD1)

3.2.2 Substrate (POME)

In this study, POME refers to palm oil sludge (POS) collected directly from separator sludge at Malaysian Palm Oil Board's (MPOB) Experimental Palm Oil Mill, Labu, Negeri Sembilan, Malaysia, was used as substrate. The fresh POME kept in the cold room for a day (to settle the solid particles) was centrifuged at 2300 *g* for 20 min at 4 ℃ using Beckman J2-M1. The supernatant was dispensed into 2 L plastic containers and then frozen at -20 ± 2 ℃. The frozen supernatant was thawed at room temperature when needed and filtered through 25 µm pore size Whatman paper to further remove solid particles. The pH of the supernatant was 4.55.

3.2.3 Mass culture of phototrophic bacteria

3.2.3.1 Inoculum preparation

The inoculum was prepared in 112 medium (Appendix I). For the marine PB species (PD1), 30 g of sodium chloride (NaCl) was added to the medium. The pH was

adjusted to 7.0 and then dispensed into 20 mL McCartney bottles. The bottles were autoclaved at 120 ℃ and 15 psi for 15 min.

A loopful of 96 h pure culture of each bacterial species was separately inoculated into each of the several autoclaved 112 medium. The bottles were incubated in anaerobic-light condition at continuous illumination of 2.0 Klux or 27.0 μmol m^{-2} s^{-1} (74 lux = 1 μmol m^{-2} s^{-1}) with 100 watts tungsten bulbs and at 30 ± 2 ℃. A 48 h bacterial culture was used as an inoculum in the following studies, unless otherwise stated.

3.2.3.2 The effect of media on growth, biomass and carotenoid production of PB

Both medium 112 and 25% POME (v/v) in distilled water was adjusted to pH 7 and evaluated for bacterial growth. For the marine isolate, 30 g of NaCl was added to either 112 medium or diluted POME. The media was then dispensed into 1 L Schott bottles and autoclaved at 120 ℃ and 15 psi for 15 min. Each bottle containing 900 mL of medium was inoculated with 10% (v/v) of 48 h inoculum as prepared above. The inoculated bottles were incubated anaerobically under continuous illumination light of 2.0 Klux with 100 watts tungsten bulbs and at 30 ± 2 ℃. At intervals of 12 h, 45 mL (for triplicates) of the culture was collected from each inoculated bottle for biomass and total carotenoids analysis.

3.2.3.2.1 Proximate analysis of biomass of PB grown in 112 medium or POME

Appropriate samples of PB were harvested at 60 h of cultivation (Figure 3.2). The bacterial samples were washed several times with sterilized 0.9% (v/v) saline solution. The samples were then immediately freeze-dried. The proximate analysis included protein, lipid, carbohydrate, ash, moisture, energy, fatty acid and amino acid profiles. However, raw POME was not characterised since the reported chemical

composition of raw POME did not vary significantly with time of production (see Appendix IIa – IIc, pages 203 - 204; Table 2.3, page 35; Table 2.4, page 36). Although POME composition is expected to vary with every batch of the processed fresh fruit bunch and time of collection, its fatty acids namely, α-linolenic acid (LNA), arachidonic acid (ARA) and eicosapentaenoic acid (EPA) which are vital for survival and growth of fish larvae did not vary significantly with time of production (see Appendix IIb, page 203).

Protein, lipid, ash and moisture content were analyzed using standard methods of AOAC:981.10, AOAC:991.36, AOAC923.03 and AOAC:950.46, respectively (Association of Official Analytical Chemists – AOAC, 1995), whereas carbohydrate was analyzed using the method as described by Pomeranz and Meloan (1987). The fatty acid methyl esters (FAME) were prepared using International Union of Pure and Applied Chemistry (IUPAC) method 2.301 (Standard methods for analysis of oils, fats and derivates, International Union of Pure and Applied Chemistry, IUPAC method 2.301, 1987). Fatty acid profiles of freeze dried samples were determined using gas chromatography on a HP5890, while the amino acid profiles of the samples were analyzed using Waters AccQ•Tag™ method (Waters, USA) and run using high-performance liquid chromatography (HPLC).

Figure 3.2 Growth of phototrophic bacteria (PB) in two different substrate media under anaerobic-light condition. A = PB grown in 112 medium; B = PB grown in POME

3.2.3.3 Analytical techniques

The dry weight (DW) of bacterial cell biomass (g/L) was determined according to Sawada and Rogers (1977) (Appendix III), while the total carotenoids (mg/g) were estimated according to Jensen and Jensen (1971) (Appendix IV). Daily growth rate was calculated by taking the difference between the final DW and initial DW and dividing it with the number of days of culture.

3.2.4 Evaluation of biomass of PB grown in 112 medium or POME as live microbial feed for rotifer culture

The three bacterial species namely B1, PD1 and KS grown in either 112 medium or diluted POME medium containing 25% POME (v/v) and 75% distilled water were used as live microbial feed for rotifer culture. The stock of rotifer *Brachionus rotundiformis* (S type) (Figure 3.3) was obtained from the Department of Fisheries Malaysia. Seventy five rotifers/mL were cultured in an inverted amber bottle with conical bottom (for easy removal of particles) (Helland et al., 1996) (Figure 3.4) and working volume of 3 L of 10 ppt salinity water. This salinity was previously tested to be

the best among 10 ppt, 15 ppt and 20 ppt salinity but comparable with 5 ppt salinity for rotifer production (see Table 8.3, page 156). Seawater was diluted to the required salinity by mixing aged fresh water before UV-irradiation. Throughout the culture period, 50% water replacement was done daily. The rotifers were fed thrice a day and each treatment was executed in triplicates. Bacteria were fed directly to the rotifers in liquid form, prepared by measuring the required wet weight of bacterial biomass (15 g) and suspending the cells in 1 L of autoclaved 10 ppt salinity water to give a feed stock containing 1.68 g DW feed/L. For each tank of 3 L culture water, rotifers were fed a total daily ration of 50 mL of the aqueous feed stock equivalent to approximately 0.084 g DW of feed biomass. Rotifer density (ind/mL) was counted daily under microscope and three readings were taken from each culture tank. Rotifers fed with b112-PD1 and bPOME-PD1, respectively were harvested at 96 h for proximate analysis. The rotifer samples were rinsed several times with filtered distilled water to discard salt, then immediately freeze-dried before analysis was carried out.

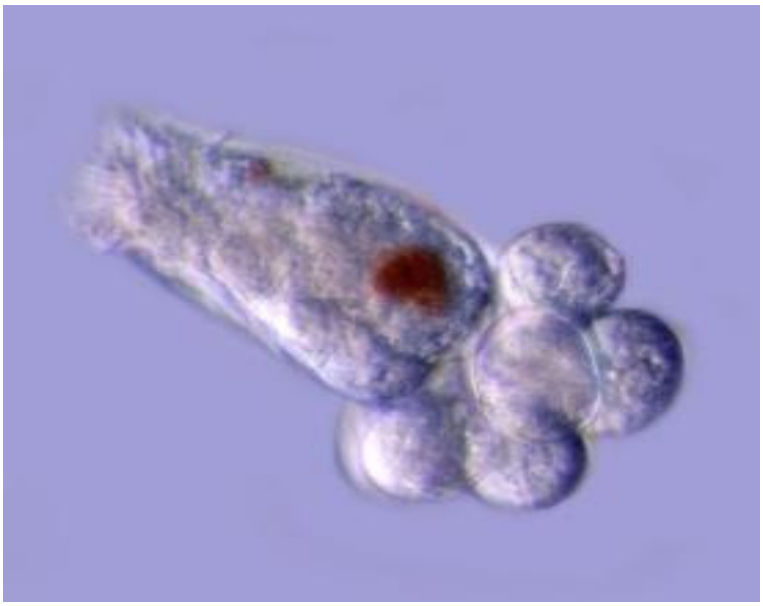


Figure 3.3 *Brachionus rotundiformis*

Figure 3.4 Rotifer and larval marble goby culture tanks

3.2.5 Studies on survival of fish larvae given live feed (rotifers and *Artemia* nauplii) fed with phototrophic bacteria (PB)

3.2.5.1 Feeding protocol

The rotifers were first filtered through 200 µm- and then 40 µm-mesh nettings to discard the rotifer culture water and unwanted debris prior to larval fish feeding. The biomass of PB grown in 112 medium or POME was separately fed to one day post-hatch (1 dph) *Artemia* nauplii. The commercially-produced *Artemia* cysts (Hong Da *Artemia* cysts, China) were used. After two days of feeding, the nauplii were harvested using a 150 µm-mesh netting. They were then rinsed twice with sterilized seawater before feeding to larval fish. The rotifers fed with PB were given to fish larvae at the rate of 10 ind/mL (Day 0 - Day 20) and 5 ind/mL (Day 21 - Day 30), while the *Artemia* nauplii fed with PB were given as additional feed from Day 21 onwards at a density of 5 ind/mL (modified feeding protocol based on Senoo et al., 1994c). Fish larvae were fed twice daily with triplicates per each treatment.

A fish density of seventeen 1 dph marble goby larvae per litre of water was used in all the larval feeding trials. The trials were executed in 4 L inverted amber bottles with conical bottoms and a working volume of 3 L of 10 ppt salinity water. This salinity was previously tested to be the best among freshwater, 5 ppt, 10 ppt, 15 ppt, 20 ppt and

30 ppt salinity (Senoo et al., 2008). Throughout the rearing period of 30 days, 30% (v/v) of water replacement was done at every 2-day intervals. The live feed density in the larvae culture was checked every day using a 1 mL Stempel pipette so as to maintain a density of 5 to 10 ind/mL. The live feed were cultured in 4 L inverted amber bottles with conical bottom and maintained at 10 ppt salinity.

3.2.5.2 Evaluation of rotifers and *Artemia* nauplii fed with bacterial biomass as live feed for fish culture

Two feeding trials using bacteria-fed rotifers and *Artemia* nauplii as live feed were conducted to evaluate the survival of fish larvae. In the first trial, fish larvae were given live feed fed with biomass of PB grown in 112 medium or live feed fed with biomass of PB grown in POME. Bacterial biomass namely of B1 and KS grown in 112 medium or POME were fed to live feed prior to larval feeding. In the second trial, larvae were given live feed fed with bPOME-B1 or bPOME-PD1 or bPOME-KS. The survival of larvae was determined by counting all the surviving larvae in the tank after completely draining off the culture water at 10-day intervals. After enumeration, the larvae were returned to their culture tanks and the same initial culture conditions were maintained.

3.2.6 Statistical analysis

The mean DW of biomass and total carotenoids of PB, mean rotifer density and mean survival of larval fish were calculated. The percent survival of larvae (Day 10, 20 and 30) were arcsine-transformed before parametric testing. Two-way ANOVA and posthoc Tukey HSD test were used and statistical analysis was done using the computer software Statistica, version 9. Principal components analysis (PCA) as a multivariate

procedure was used to interpret the amino acid and fatty acid profiles of the PB. PCA was run on the software CANOCO 4.5 (Ter Braak & Smilauer, 2002).

3.3 Results

3.3.1 Bacterial growth studies

3.3.1.1 The effect of media on the production of bacterial biomass and carotenoids

Both media, 112 medium and POME supported the growth of the three species of PB (Figure 3.5). The growth of all species of bacteria in the two media did not show exponential growth phase within 3 days of culture when the data were plotted on normal or semi-log plot (not shown) except 112-KS; most of the bacteria showed a gradual increase in biomass until 72 h (Figure 3.5). Only 112-KS appeared to show exponential growth at 60 h. Therefore, the average daily growth rate rather than the specific growth rate was used as the measure of bacterial growth in this study.

The bacterial biomass and daily growth rate of the three species of PB grown in 112 medium ranged from 1.44 to 3.34 g/L and 0.48 to 1.11/d, respectively. The rates were higher when compared to PB grown in POME (0.29 – 1.91 g/L; 0.10 – 0.64/d) (Table 3.1). Thus, the type of medium had a significant effect ($P < 0.01$) on the bacterial biomass production and growth (Appendix Va). Overall, the PB grown in 112 medium had significantly higher ($P < 0.01$) daily growth rate when compared to growth rate of PB grown in POME regardless of bacterial species tested (Table 3.1).

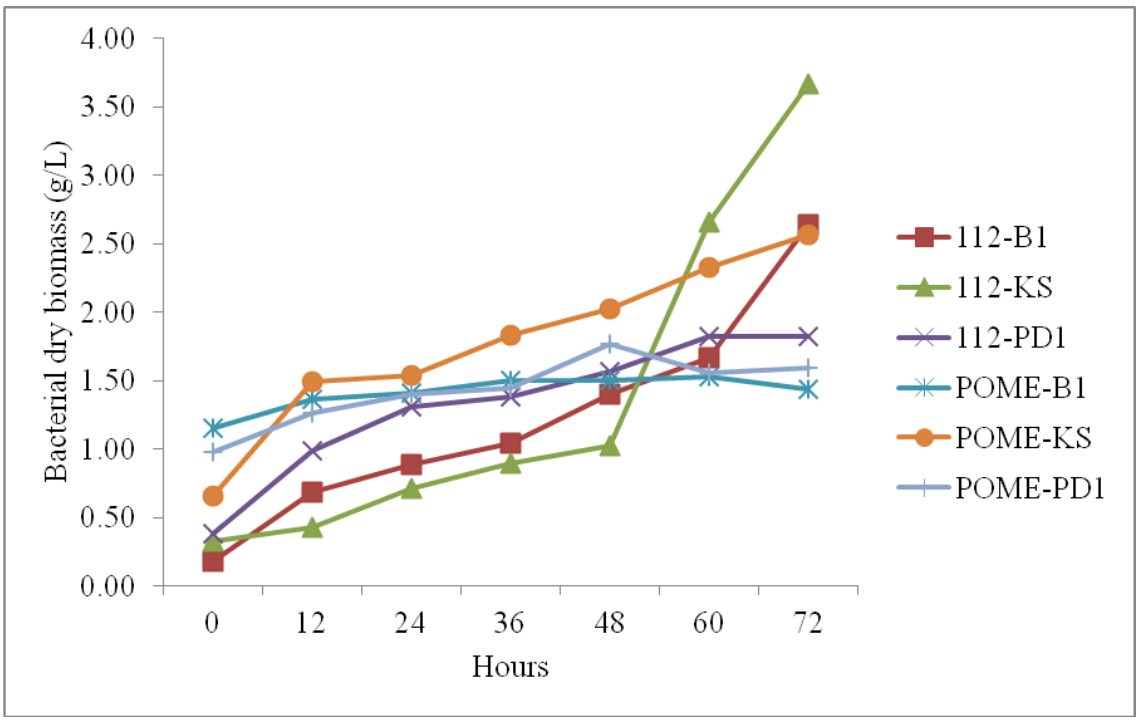


Figure 3.5 Biomass production of phototrophic bacteria grown in 112 medium or POME (30 ± 2 ℃; light intensity of 2.0 Klux; pH 7) (data points – mean of triplicate values)

Table 3.1 Maximum dry weight (DW) of cell biomass, average daily growth rate (over 3 days) and total carotenoids of phototrophic bacteria grown in 112 medium or POME (30 ± 2 ℃; light intensity of 2.0 Klux; pH 7)

Treatments	Dry cell biomass[1] (g/L) at 0 h	Dry cell biomass[1] (g/L) at 72 h	Maximal dry cell biomass[1] (g/L)	Daily growth rate[1] (d^{-1})	Total carotenoids[1] (mg/g) at 0 h	Total carotenoids[1] (mg/g) at 72 h
112 medium						
B1	0.18 ±0.00	2.64 ±0.37	2.46 ±0.37	0.82	5.56 ±0.00	0.94 ±0.14
PD1	0.38 ±0.00	1.82 ±0.21	1.44 ±0.21	0.48	2.95 ±0.00	0.94 ±0.18
KS	0.33 ±0.00	3.67 ±0.07	3.34 ±0.07	1.11	3.77 ±0.00	0.54 ±0.16
POME						
B1	1.15 ±0.00	1.44 ±0.05	0.29 ±0.05	0.10	1.51 ±0.00	1.38 ±0.25
PD1	0.98 ±0.00	1.59 ±0.14	0.61 ±0.14	0.20	0.34 ±0.00	0.17 ±0.13
KS	0.66 ±0.00	2.57 ±0.07	1.91 ±0.07	0.64	2.32 ±0.00	0.94 ±0.07

[1] Mean of triplicate values

Further, time also had an effect on bacterial biomass production (Appendix Va). In all samples, the bacterial dry cell biomass increased with incubation time except POME-B1 and POME-PD1 as their biomass production started to decrease after 60 h. It

was also observed that increasing the incubation time to 72 h did not significantly increase biomass production as compared to biomass production at 60 h (Figure 3.5).

Apart from that, the culture medium also had an effect on total carotenoids (Appendix Vb). The biomass of PB grown in 112 medium had significantly ($P < 0.01$) higher mean total carotenoids compared to biomass of PB grown in POME (Table 3.1). Overall, B1 was the best carotenoid producer regardless of culture medium tested. Further, the amount of total carotenoids decreased as time of incubation proceeded (Figure 3.6). Thus, time had a significant effect ($P < 0.01$) on the production of carotenoids (Appendix Vb).

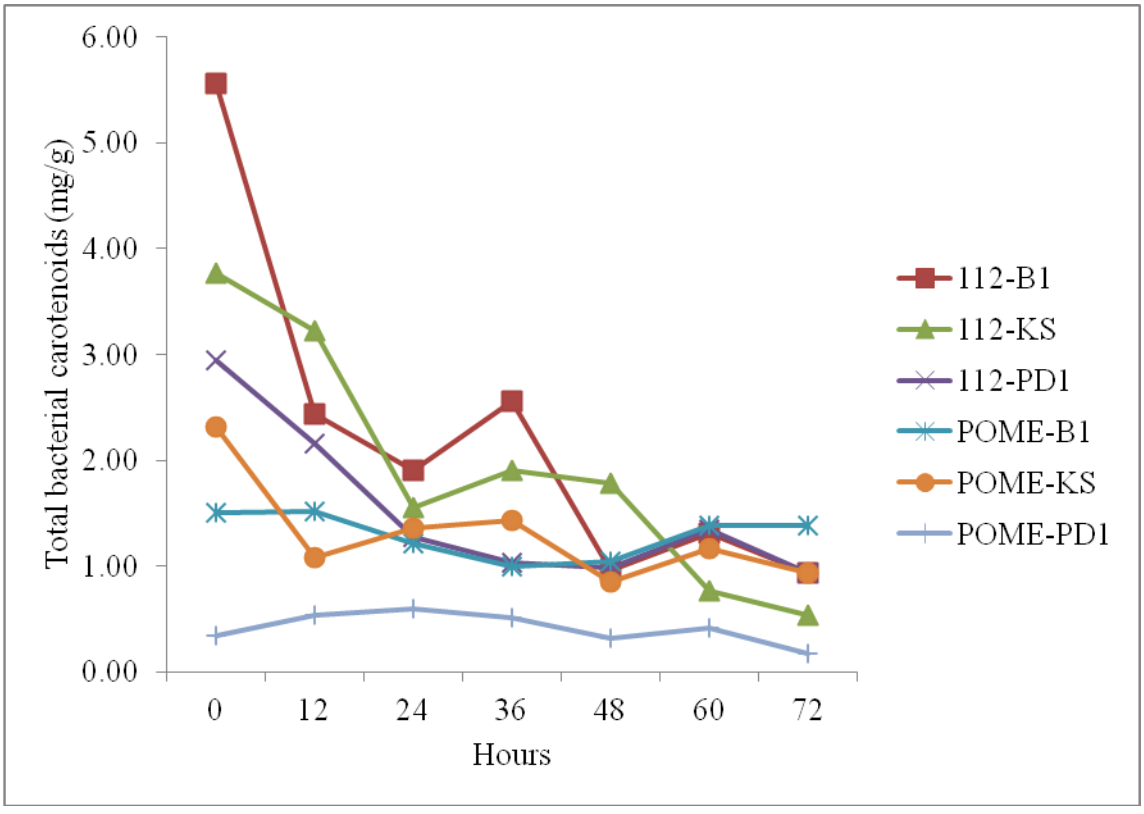


Figure 3.6 Total carotenoids of phototrophic bacteria grown in 112 medium or POME (30 ±2 ℃; light intensity of 2.0 Klux; pH 7) (data points – mean of triplicate values)

3.3.2 Production of rotifer using phototrophic bacteria (PB) as live microbial feed

3.3.2.1 Evaluation of biomass of PB grown in 112 medium or POME as live microbial feed for rotifer culture

The highest rotifer density was harvested when b112-B1 was the live microbial feed, while the lowest density was obtained with bPOME-PD1 as feed. In brief, when the biomass of PB grown in 112 medium was given to rotifers, the density was significantly higher ($P < 0.01$) than when the rotifers were fed with biomass of PB grown in POME (Table 3.2; Appendix VI).

Further, rotifers reached peak population density or exponential growth phase at Day 3 based on statistical analysis. In most treatments, rotifer density decreased after Day 4 (Table 3.2). Hence, the day of culture had a significant effect ($P < 0.01$) on rotifer mass culture. There was also significant interactive effect ($P < 0.01$) of type of feed and day of culture on rotifer production where rotifers fed with b112-B1 had the highest rotifer density (395 ind/mL) at Day 3 as compared to other treatments (Table 3.2).

Table 3.2 Mean rotifer density (ind/mL) fed with bacterial biomass and cultured in 10 ppt salinity water

Treatments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
112 medium[1]							
B1	75 ± 0^{c}	18 ± 3^{c}	113 ± 25^{bc}	395 ± 30^{a}	248 ± 41^{abc}	91 ± 14^{bc}	39 ± 3^{c}
PD1	75 ± 0^{c}	58 ± 10^{c}	127 ± 12^{bc}	147 ± 12^{abc}	125 ± 14^{bc}	92 ± 18^{bc}	13 ± 14^{c}
KS	75 ± 0^{c}	38 ± 11^{c}	225 ± 139^{abc}	198 ± 283^{abc}	145 ± 223^{abc}	16 ± 31^{c}	0^{c}
POME[1]							
B1	75 ± 0^{c}	32 ± 11^{c}	129 ± 87^{bc}	198 ± 45^{abc}	246 ± 24^{abc}	332 ± 19^{ab}	225 ± 88^{abc}
PD1	75 ± 0^{c}	50 ± 3^{c}	102 ± 3^{bc}	94 ± 21^{bc}	72 ± 12^{c}	15 ± 13^{c}	0^{c}
KS	75 ± 0^{c}	21 ± 19^{c}	96 ± 89^{bc}	113 ± 178^{bc}	107 ± 173^{bc}	27 ± 43^{c}	5 ± 9^{c}

[1] Mean of nine replicate values; mean rotifer density that does not share a common superscript letter differ significantly ($P < 0.01$); see Appendix VI for detailed statistical test of significance

3.3.3 Studies on survival of fish larvae on a diet of phototrophic bacteria (PB)-fed live feed (rotifers and *Artemia* nauplii)

3.3.3.1 The effect of live feed fed with biomass of PB grown in POME or 112 medium on survival of marble goby larvae

Fish larvae on a diet of live feed which had been fed with bPOME-B1 and bPOME-KS survived until the end of feeding trial (Day 30). Larvae given live feed fed with bPOME-B1 and bPOME-KS had significantly higher ($P < 0.01$) survival than the larvae given live feed fed with b112-B1 and b112-KS (Table 3.3; Appendix VII).

Table 3.3 Mean survival of marble goby larvae on a diet of live feed fed with *Rhodopseudomonas palustris* grown in 112 medium (b112-B1) and *Rhodobacter sphaeroides* grown in 112 medium (b112-KS) or live feed fed with *Rhodopseudomonas palustris* grown in POME (bPOME-B1) and *Rhodobacter sphaeroides* grown in POME (bPOME-KS), at 10 ppt salinity water for 30 days. (Initial stocking size in 3 L = 50 larvae)

Day of culture	Live feed fed with b112-B1 and b112-KS	Live feed fed with bPOME-B1 and bPOME-KS
	% survival (from Day 0) [1]	
0	100.0 ±0.0	100.0 ±0.0
10	18.7 ±5.0^{b}	50.0 ±7.2^{a}
20	0.0^{c}	30.0 ±7.2^{b}
30	0.0^{c}	24.0 ±6.0^{b}

[1] Mean of triplicate values; % survival that does not share a common superscript letter differ significantly ($P < 0.01$); see Appendix VII for detailed statistical test of significance

Additionally, the mortality rate increased with day of culture. Thus, day of culture also had a significant effect ($P < 0.01$) on larval survival. At Day 30, larvae reared on live feed fed with bPOME-B1 and bPOME-KS had 24.0% survival and this was significantly higher ($P < 0.01$) than the larvae given live feed fed with b112-B1 and b112-KS (0%) (Table 3.3).

3.3.3.2 The effect of live feed fed with biomass of three species of PB grown in POME on survival of marble goby larvae

Fish larvae on a diet of live feed fed with bPOME-PD1 survived until the end of 30 days feeding period. The highest larval survival was observed in those given live feed fed with bPOME-PD1; followed by those given live feed fed with bPOME-KS and live feed fed with bPOME-B1 (Table 3.4). Hence, feed type had a significant effect ($P < 0.01$) on survival of larvae (Appendix VIII).

Table 3.4 Mean survival of marble goby larvae on a diet of live feed fed with biomass of three species of phototrophic bacteria grown in POME, at 10 ppt salinity water for 30 days. (Initial stocking size in 3 L = 50 larvae)

Day of culture	Live feed fed with bPOME-B1	Live feed fed with bPOME-PD1	Live feed fed with bPOME-KS
	% survival (from Day 0) [1]		
0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
10	8.0 ±20.5^{ab}	30.7 ±34.1^{a}	10.7 ±15.1^{ab}
20	0.0^{c}	14.6 ±27.9^{ab}	0.0^{c}
30	0.0^{c}	13.6 ±25.8^{ab}	0.0^{c}

[1] Mean of triplicate values; % survival that does not share a common superscript letter differ significantly ($P < 0.01$); see Appendix VIII for detailed statistical test of significance

Moreover, the number of larvae decreased with day of culture. Thus, it was shown that day of culture also had a significant effect ($P < 0.01$) on larval survival. However, interactive effects of type of feed and day of culture were not observed ($P > 0.05$) on larval survival. At Day 30, the larvae on a diet of live feed fed with bPOME-PD1 had 13.6% survival and it was significantly greater that other two treatments tested (0%) (Table 3.4).

3.3.4 Fatty acid and amino acid profiles of freeze-dried phototrophic bacteria (PB) biomass and rotifers

Although PB grown in 112 medium grew faster and had higher amounts of protein than PB grown in POME, only bPOME-PD1 contained both EPA and DHA

which are essential for larval survival and growth (Table 3.5; Appendix IXa). Further, the percentage of total SFA in biomass of PB grown in POME was significantly higher than biomass of PB grown in 112 medium (Table 3.5). In contrast, the former treatments had slightly lower percentage of total MUFA and PUFA as compared to the latter treatments. bPOME-KS recorded the lowest amount of PUFA (16.88%), while bPOME-B1 had the highest PUFA (54.29%) as compared to other treatments (Table 3.5). In addition, the total percentage of essential amino acids was only slightly greater than non-essential amino acids in all bacterial samples (Table 3.5; Appendix IXb). On the other hand, the nutritional profile of rotifers fed with bPOME-PD1 was comparable to those fed with b112-PD1 except the former had greater ARA, DHA and EPA than the latter (Table 3.5; Appendix XVIII).

Further, biplots of the samples and the types of fatty acids and amino acids they contained were derived from PCA (Figure 3.7). The first and second PCA axis explained 43.2% and 31.7% of the total variation, respectively. The bPOME-B1 contained higher amounts of LNA (linolenic acid, C18:3n-3), ARA and EPA, whereas the b112-PD1 and bPOME-PD1 contained significant amounts of DHA and LNA. The b112-KS had significant amount of LA (linoleic acid, C18:2n-6c) and EPA, while bPOME-KS had higher amount of LA and LNA (Figure 3.7; Appendix IXa).

Table 3.5 Proximate composition (%), fatty acids (% total fatty acids) and amino acids (% protein) of the biomass of three species of freeze-dried phototrophic bacteria (B1, KS, PD1) and rotifers (mean of duplicate values). Bacteria were cultured in either synthetic 112 medium or POME.

Proximate profile	b112-B1	bPOME-B1	b112-KS	bPOME-KS	b112-PD1	bPOME-PD1	Rotifers fed with b112-PD1	Rotifers fed with bPOME-PD1
Proximate composition (%)								
Protein	61.6	36.0	59.9	51.1	63.7	44.3	42.2	47.6
Lipid	8.6	5.8	7.1	6.1	4.4	7.4	12.3	6.7
Carbohydrate	5.4	10.2	4.3	8.4	8.1	7.4	6.9	5.5
Ash	15.7	42.0	19.3	26.8	13.2	33.4	11.0	10.6
Moisture	8.7	6.0	9.4	7.6	10.6	7.5	27.6	29.6
Energy, Kcal/100g	345 (1449kJ)	237 (995kJ)	321 (1348kJ)	293 (1231kJ)	327 (1373kJ)	273 (1147kJ)	308 (1294kJ)	272 (1142kJ)
Fatty acids								
ARA	0.00	10.32	0.00	0.00	0.00	0.00	0.00	0.66
EPA	0.00	18.01	29.07	1.03	0.00	3.94	0.90	2.59
DHA	0.00	0.00	0.00	0.00	0.80	2.60	0.79	1.20
Total SFA	16.93	32.95	29.05	46.98	53.64	45.42	36.54	41.20
Total MUFA	53.56	12.76	27.62	36.14	18.92	11.75	33.30	40.93
Total PUFA	28.32	54.29	43.33	16.88	27.44	42.83	30.16	17.87
Percentage total fatty acids	98.81	100.00	100.00	100.00	100.00	100.00	100.00	100.00
n-3 PUFA	0.00	22.49	30.33	3.36	3.65	10.73	2.76	5.44
n-6 PUFA	15.40	18.23	11.85	7.25	9.91	18.19	23.08	12.02
ARA/EPA	-	0.57	0.00	0.00	-	0.00	0.00	0.26
DHA/EPA	-	0.00	0.00	0.00	-	0.66	0.88	0.46
DHA/ARA	-	0.00	-	-	-	-	-	1.81

Table 3.5, continued

Proximate profile	b112-B1	bPOME-B1	b112-KS	bPOME-KS	b112-PD1	bPOME-PD1	Rotifers fed with b112-PD1	Rotifers fed with bPOME-PD1
Amino acids								
Total essential amino acids	26.44	15.78	25.55	18.79	24.88	19.56	21.41	20.89
Total non-essential amino acids	24.56	14.91	22.82	17.96	23.94	18.63	24.54	23.58
Percentage total amino acids	51.00	30.69	48.37	36.74	48.82	38.19	45.95	44.47

See Appendix IX and Appendix XVIII for detailed statistical test of significance

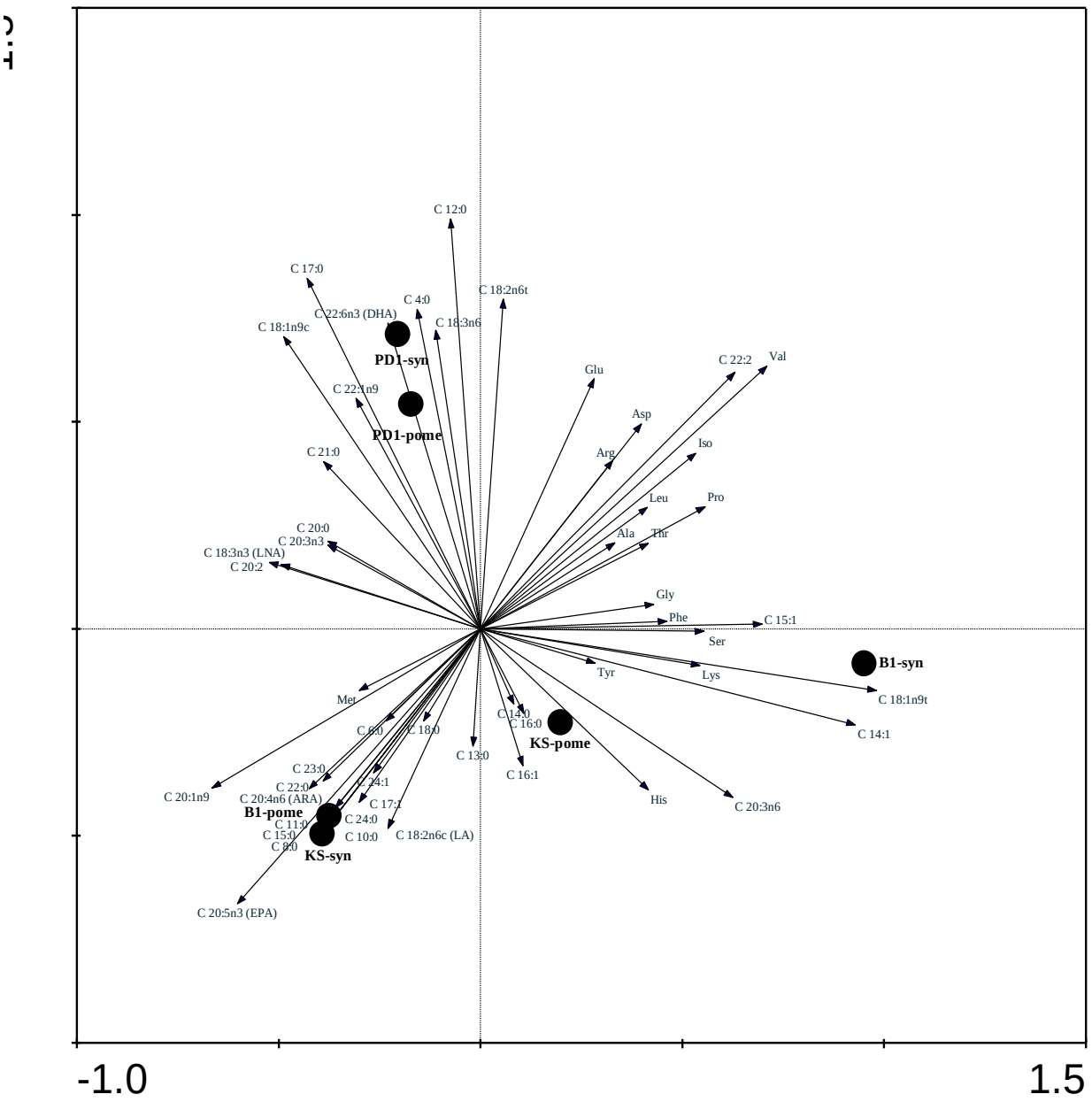


Figure 3.7 PCA of the fatty acid compositions of freeze-dried bacterial biomass. Samples (filled circles): B1-syn = b112-B1; PD1-syn = b112-PD1; KS-syn = b112-KS; B1-pome = bPOME-B1; PD1-pome = bPOME-PD1; KS-pome = bPOME-KS; Variables (arrows): 37 fatty acids and 16 amino acids in conventional notations

3.4 Discussion

3.4.1 The effect of substrate on the growth of phototrophic bacteria (PB)

The present study has shown that marble goby larvae survived only on live feed fed with bPOME-PD1. However, PB grown in POME, whether POME-B1, POME-KS or POME-PD1 had significantly lower ($P < 0.01$) daily growth rate as compared to PB

grown in 112 medium (112-B1, 112-KS, 112-PD1). This is likely due to the culture medium itself. POME is naturally dark brown and contains a high amount of suspended particles (Ma, 2000). Under anaerobic-light condition, this substrate reduced the transmission of light, whereas the 112 medium is clear yellow and allows better transmission of light through it. Consequently, the latter gave better cell growth. Although bacterial cells multiplied slower in POME than in 112 medium, the bacteria could grow in POME which had a high organic load (Phang, 1990; Alias & Tan, 2005). However, a small difference in biomass production rate was observed between all bacteria cultures after 60 h of incubation. This is likely due to the limited nutrients available in the substrate and minimal light transfer as cell concentrations increased. Thus, PB were harvested at 60 h of cultivation in subsequent studies, unless otherwise stated. The main reason for harvesting the bacteria at 60 h is because there was not significantly different between the bacterial biomass harvested at 60 h and 72 h of cultivation. This was determined based on the statistical analysis (see Appendix Va-ii, page 208). Another reason for bacterial harvest just before the exponential phase of growth is to avoid the risk of culture collapse because exponential phase of growth of these bacteria fall between 3 - 4 days of cultivation (Maheswari, 1997; Getha et al., 1998a; Azad et al, 2003).

Rhodobacter sphaeroides (KS) was observed to be capable of growing well in both media as compared to other tested bacterial species (see Table 3.1). Basically, the differences in mass culture among bacterial species are due to the light illumination condition, concentration and type of substrate and the species of PB (Kim & Lee, 2000). However, the efficacy of KS as aquaculture feed needs further investigation. Commonly, bacterial biomass grown in waste is a mixture of bacteria and culture substrate. In this study, the amount of substrate particles in 1 L of 25% diluted filtered POME (v/v) was estimated to be 0.438 ± 0.006 g DW ($n = 5$). A high amount of solid debris in "bacterial

biomass" is not advisable as larval food as it might deteriorate the culture water quality and encourage the growth of pathogens which could lead to larval mortality (Simon, 1978). The separation of pure bacterial biomass from the waste substrate is somehow difficult although repeated washings of the cell biomass with 0.9% saline (v/v) during harvesting could minimize the contamination (Azad et al., 2001).

Further, the reduction of total carotenoids in bacteria as time of incubation proceeded could be due to the disintegration, isomerization or dehydration of carotenoids, particularly with the continuous light illumination (Britton & Young, 1993). In this study, the highest total bacterial carotenoids were obtained at 0 h. The reduction of total bacterial carotenoids with time could also be due to the high light intensity provided in this study which suppressed the production of carotenoids (Shipman et al., 1977). This problem can be overcome by applying continuous mixing in bacteria culture as it favours the conversion efficacy of carbon sources in waste, distributes the heat diversion within the culture medium and avoid cell flocculation (Kim et al., 1982). For instance, an increase in total carotenoid contents of bacteria to 2.5 mg/g dry biomass after 4 days of treatment at low-speed mixing was reported (Getha, 1995). The total carotenoids of bacteria, however, demonstrated a reverse correlation with bacterial biomass production (see Table 3.1).

3.4.2 The biochemical composition of waste-grown phototrophic bacteria (PB)

Selection of the right PB species and strain for aquaculture purposes is not only based on their capability of growing fast but also their nutritional profile. PB generally lack PUFA and HUFA due to their minimal variability in lipid compositions and fatty acids (Imhoff & Imhoff, 1995). Their fatty acid profile is basically influenced by cultivation medium (Imhoff & Imhoff, 1995). Thus, the use of right substrate to culture the bacteria is crucial for larviculture. For instance, PD1 grown in sardine processing

wastewater had low contents of PUFA and no HUFA despite having more than 95% C-14, C-16 and C-18 straight chain SFA and MUFA. This low quality feed had affected the growth and survival of penaeid shrimp larvae (Azad et al., 2002). In the present study, POME-B1 and POME-KS both lacked DHA. In contrast, POME-PD1 contained significant amounts of EPA and DHA (see Table 3.5; Figure 3.7). The nutritional profiles of these PB grown in POME were, however, still superior to PB grown in 112 medium. 112-KS contained only EPA, whereas 112-PD1 contained only DHA. 112-B1 had none of the EFA mentioned above (see Table 3.5; Figure 3.7). Indirectly, this study had shown that PD1 and B1 are able to incorporate the nutrients from POME into their cells based on the presence of high amount of EPA in bPOME-B1 and bPOME-PD1 which were not found in b112-B1 and b112-PD1. On the other hand, KS has limited ability to uptake the nutrients from POME (see Table 3.5; Figure 3.7). Further, some bacteria are reportedly able to produce PUFA (Ratledge, 2001). For instance, PB such as *Rhodopseudomonas* spp. are able to produce DHA (Singh & Ward, 1997). Thus, the presence of significant amount of DHA in bPOME-PD1 showed that PD1 is able to biosynthesis DHA because raw POME does not contain DHA (see Appendix IIb, page 203). All the three important fatty acids namely oleic acid (LA), linoleic acid and α-linolenic acid (LNA) which are the basic precursors to the biosynthesis PUFA (Milan & Sakayu, 1999) were only found in PD1 whereas B1 and KS lacked one of these fatty acids (see Figure 3.7). Although B1 and KS grown in POME contained LNA, they appeared to have limited ability to biosynthesis PUFA because DHA was not found in them. However, they contained significantly higher amount of EPA (see Figure 3.7) when compared to their substrate (POME) (see Appendix IIb, page 203). The higher amount of EPA in bPOME-B1 as compared to bPOME-KS and bPOME-PD1 is likely due to the conversion of arachidonic acid (ARA) to EPA since ARA was significantly

high in bPOME-B1, whereas EPA in bPOME-KS and bPOME-PD1 was directly synthesized from LNA because ARA was not found in them (see Figure 3.7).

Methionine has been reported to be the limiting amino acid in PB (Shipman et al., 1975). However, in the present study, amino acid analysis reveals that these PB contained reasonable amounts of methionine. The amount of methionine (as % of the total protein) in the three biomasses of PB grown in POME ranged from 0.79 to 1.35%, whereas 1.38 to 3.38% in biomasses of PB grown in 112 medium. These values were only slightly lower than the guideline of Food and Agriculture Organization of the United Nations (2.20%) (FAO, 1980). Therefore, POME used as a substrate for mass culture bacteria enhances both the amino acid and fatty acid profiles.

3.4.3 The effect of phototrophic bacteria (PB) grown in POME or 112 medium on rotifer production

In this study, rotifers reproduced well when PB (B1, KS, PD1) were supplied as live microbial feed. This suggests that both biomasses of PB grown in 112 medium or POME are non-toxic to rotifers and could be used as rotifer feed. However, rotifers fed with biomass of PB grown in 112 medium had significantly higher ($P < 0.01$) density as compared to those fed with biomass of PB grown in POME. Different rotifer densities could be due to the preference of rotifers to certain bacterial species, size of the feed and feed digestibility. The right feed could improve intake and assimilation leading to higher production. Overall, B1 was superior to other bacterial species tested in term of stimulating rotifer growth, regardless of the substrate used to mass culture it. Thus, the use of B1 as a live microbial feed for rotifers may reduce the cost of rotifer production in aquaculture.

3.4.4 The effect of phototrophic bacteria (PB)-fed organisms on larval fish survival

Larval marble goby could survive on a diet of live feed (rotifers and *Artemia* nauplii) cultured from biomass of PB grown in POME, while all larvae eventually died when given a diet of live feed fed with biomass of PB grown in 112 medium (see Table 3.3). This study thus supports the hypothesis that PB grown in 112 medium lack EFA or/and their precursors which are essential for larval survival. It appears that while biomass of PB grown in 112 medium is good in production of rotifer, the rotifers produced cannot support good survival of fish larvae. Fish larvae only survived when given a diet of live feed fed with bPOME-PD1; none survived when given live feed fed with bPOME-B1 or bPOME-KS (see Table 3.4). The fatty acid profile reveals that bPOME-B1 had high amounts of ARA and EPA, whereas bPOME-KS contained low amount of EPA. On the other hand, bPOME-PD1 contains but low amounts of EPA and DHA. Hence, complete mortality suffered by fish larvae reared on rotifers fed with bPOME-B1 or bPOME-KS is likely due to the lack of DHA or excessively high amount of EFA in their diet (see Table 3.5). This interpretation is based on dietary nutrition since the nutritional value of rotifers is directly and positively correlated to the nutritional value of their diet with minimal modification (Lubzens et al., 1995). The DHA seems to be more important than ARA in sustaining larval fish survival (see Table 3.4).

In conclusion, PB can grow in POME although their growth rates appear slower compared to their growth in synthetic 112 medium. The PB grown in 112 medium or POME can be used as rotifer feed. However, marble goby larvae survived only when provided with live feed fed with bPOME-PD1. The other PB, B1 and KS, although supporting high rotifer production, cannot sustain fish survival via the live feed they fed. Hence, PD1 is selected as the best PB for scaling up in POME (Chapter 4) as it is the

most nutritious and shows promising results based on the survival of marble goby larvae and rotifer production experiments.

CHAPTER 4

MASS CULTURE OF *RHODOVULUM SULFIDOPHILUM* GROWN IN PALM OIL MILL EFFLUENT FOR AQUACULTURE

Summary of significant findings

Rhodovulum sulfidophilum (PD1) grew well in palm oil mill effluent (POME). At 60 h of cultivation, the cell dry biomass of *R. sulfidophilum* grown in POME (POME-PD1) in ziplock bags was higher than POME-PD1 cultured in Schott bottles. PD1 grew well in 25% POME at a light intensity of 1.5 Klux (20.3 μmol m^{-2} s^{-1}). The growth of PD1 was not influenced by pH and salinity. The production of bacterial carotenoids was only affected by light intensity but not by salinity and pH of the culture medium. The optimum culture conditions for mass production of POME-PD1 are, a) 25% POME (v/v); b) pH medium of 4.55; c) 30 ppt salinity; d) a light intensity of 1.5 Klux; and e) ziplock bags.

4.1 Introduction

Maximum production of a cheap yet highly nutritious live food in the shortest time is an important goal in feed production. Purple non-sulfur PB are well known to be able to grow in various substrates including industrial wastes with high organic pollution load (Kobayashi & Kobayashi, 1995). The optimization of cell yield, carotenoid, protein and lipid content of PB cultured in wastes such as cassava starch, soybean whey and pineapple waste medium has been reported (Sasaki et al., 1991). In Malaysia, growth optimization of B1 in sago starch processing wastewater and PD1 in sardine processing wastewater have been documented by Getha et al. (1998a) and Azad (2004), respectively but these waste-grown PB lack EFA such as ARA, EPA or DHA which are very important for survival of fish larvae. Hence, it is necessary to consider a good nutritional profile that is rich in PUFA and HUFA in choosing a suitable substrate

for enriching PB as an aquaculture feed. To date, there is no report on the utilization of any PB grown in POME for aquaculture purposes. The previous component study (Chapter 3) has shown that POME-PD1 contained a significant amount of DHA and EPA (see Table 3.5, page 56; Figure 3.7, page 58), and rotifers and *Artemia* nauplii fed with the bacteria can significantly improve the survival of marble goby larvae (see Table 3.4, page 54). Therefore, the conditions for the optimal production of POME-PD1 has to be further studied. Factors such as pH, salinity (Biebl & Pfennig, 1981), specific substrate, light intensity (Madigan & Gest, 1988), temperature and lamp types can affect the growth, biomass production and quality of bacteria. Thus, the main objective of this part of the study was to mass culture POME-PD1 in a suitable reactor for aquaculture. The specific aim was to optimize the culture conditions, namely POME concentration, pH, salinity and light intensity for growth of POME-PD1 in a selected reactor so as to maximize its production.

4.2 Materials and methods

4.2.1 Mass culture of *Rhodovulum sulfidophilum* grown in POME (POME-PD1)

4.2.1.1 The effects of growth parameters and type of reactor on mass culture of *Rhodovulum sulfidophilum* grown in POME (POME-PD1)

The parameters tested were a) POME concentration [25, 50, 75, 100% (v/v)]; b) pH (4.55, 7, 9); c) salinity (0, 5, 10, 20, 30 ppt); and d) light intensity [1.0 Klux (13.5 µmol m^{-2} s^{-1}), 1.5 Klux (20.3 µmol m^{-2} s^{-1}), 2.5 Klux (33.8 µmol m^{-2} s^{-1}), 3.0 Klux (40.5 µmol m^{-2} s^{-1})]. Ziplock bags (Figure 4.1) and 1 L Schott bottles (Figure 4.2) each containing 900 mL of medium were evaluated for cultivation. Ten percent of inoculum (112-PD1) and diluted POME medium were used in this study, unless otherwise stated. Each of the growth parameter was executed separately and each treatment was done in triplicates. Calibration curve of POME-PD1 (g DW against optical density readings at

660 nm) was derived by culturing the bacteria under optimal conditions determined from the above tests. In all the experiments, the bacterial biomass (Figure 4.3) was harvested at 60 h of cultivation (see Appendix Va-ii, page 208) by centrifugation at 2300 *g* for 20 min at 4 ℃ using the Hettich centrifuge.

Figure 4.1 Growth of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) in ziplock bag under anaerobic-light condition. A = Before inoculation; B = After 60 h of inoculation

Figure 4.2 Growth of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) in 1 L Schott bottle under anaerobic-light condition. A = Before inoculation; B = After 60 h of inoculation

Figure 4.3 Biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1)

4.2.1.2 Analytical techniques

The DW of bacterial cell biomass (g/L) was determined according to Sawada and Rogers (1977) (Appendix III), while the total carotenoids (mg/g) were estimated according to Jensen and Jensen (1971) (Appendix IV).

4.2.2 Statistical analysis

The mean DW of biomass and total carotenoids of POME-PD1 were calculated. Two-way ANOVA and posthoc Tukey HSD test were used and statistical analysis was done using the computer software Statistica, version 9.

4.3 Results

4.3.1 The effects of different POME concentrations and type of reactors on the production of biomass of *Rhodovulum sulfidophilum* (PD1)

The production of PD1 bacterial biomass was affected by POME concentration (Appendix Xa). Bacteria grown in 25% POME (v/v) had significantly higher ($P < 0.01$) cell dry mass compared to bacteria grown in other POME concentrations tested

irrespective of type of reactor used. The bacterial growth was suppressed in higher concentrations of POME as compared to growth in 25% POME. However, there was no significant difference in cell dry mass when bacteria were grown in other POME concentrations tested (50%, 75%, 100%) (Table 4.1a).

Table 4.1 Mass culture of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) at 60 h of cultivation (30 ± 2 ℃)

Growth parameters	Dry cell biomass (g/L)		Total carotenoids (mg/g)	
a) POME concentration[1] (pH 7; 30 ppt; light intensity of 2.0 Klux)	Ziplock bag	Schott bottle	Ziplock bag	Schott bottle
25%	1.64 ± 0.64^{a}	0.43 ± 0.03^{b}	-	-
50%	0.40 ± 0.05^{b}	0.27 ± 0.42^{b}	-	-
75%	0.24 ± 0.23^{b}	0.32 ± 0.04^{b}	-	-
100%	0.39 ± 0.05^{b}	0.13 ± 0.23^{b}	-	-
b) pH[1] (25% POME (v/v); 30 ppt; light intensity of 2.0 Klux)				
4.55	1.52 ± 0.81^{a}	1.46 ± 0.42^{a}	0.90 ± 0.58^{h}	0.85 ± 0.48^{h}
7.00	1.50 ± 0.11^{a}	1.42 ± 0.12^{a}	1.05 ± 0.07^{h}	1.17 ± 0.07^{h}
9.00	1.67 ± 0.10^{a}	1.18 ± 0.09^{a}	1.06 ± 0.09^{h}	1.21 ± 0.11^{h}
c) Salinity[1] (ppt) (25% POME (v/v); pH 4.55; light intensity of 2.0 Klux)				
0	1.53 ± 0.08^{a}	1.42 ± 0.07^{a}	1.78 ± 1.37^{h}	0.17 ± 0.05^{i}
5	1.59 ± 0.24^{a}	1.62 ± 0.15^{a}	1.04 ± 0.28^{hij}	0.25 ± 0.12^{ij}
10	1.55 ± 0.10^{a}	1.49 ± 0.09^{a}	0.92 ± 0.08^{hij}	0.76 ± 0.41^{hij}
20	1.49 ± 0.07^{a}	1.66 ± 0.25^{a}	1.07 ± 0.10^{hj}	0.74 ± 0.27^{hij}
30	1.67 ± 0.03^{a}	1.57 ± 0.09^{a}	1.26 ± 0.61^{h}	0.83 ± 0.29^{hij}
d) Light intensity[1] (Klux) (25% POME (v/v); pH 4.55; 30 ppt)				
1.0	7.48 ± 6.62^{a}	6.35 ± 2.65^{a}	0.85 ± 1.25^{h}	0.27 ± 0.11^{h}
1.5	14.28 ± 3.31^{a}	5.21 ± 2.95^{a}	0.12 ± 0.04^{h}	0.47 ± 0.23^{h}
2.5	3.87 ± 4.16^{a}	1.22 ± 0.09^{a}	0.70 ± 0.46^{h}	1.45 ± 0.42^{h}
3.0	2.58 ± 1.33^{a}	1.18 ± 0.27^{a}	0.71 ± 0.22^{h}	1.39 ± 0.54^{h}

[1] Mean of triplicate values; mean dry cell biomass and total carotenoids that do not share a common superscript letter differ significantly ($P < 0.01$); see Appendix X, XI, XII and XIII for detailed statistical test of significance

On the other hand, ziplock bag favoured bacterial growth when compared to Schott bottle. The bacterial biomass obtained from ziplock bag was significantly higher ($P < 0.05$) than from Schott bottle regardless of POME concentrations tested (Table 4.1a).

4.3.2 The effects of different pH and type of reactors on the production of biomass and carotenoids of *Rhodovulum sulfidophilum* grown in POME (POME-PD1)

pH of culture medium and type of reactor had no effects on production of bacterial biomass (Appendix XIa). At 60 h, the highest mean DW of biomass of 1.67 g/L was obtained from POME-PD1 cultured in ziplock bag with pH medium of 9, while the lowest mean DW of biomass of 1.18 g/L was harvested from bacteria grown in Schott bottle with pH medium of 9 (Table 4.1b). Overall, bacteria grown in ziplock bag had slightly higher biomass than bacteria grown in Schott bottle under comparable pH although statistical analysis showed no significant difference ($P < 0.05$) (Table 4.1b).

Further, pH and type of reactor also did not affect the production of bacterial carotenoids (Appendix XIb). The highest total carotenoids of 1.21 mg/g were reported from bacteria cultured in Schott bottle with pH medium of 9 (Table 4.1b). There was no correlation between bacterial biomass and total carotenoids produced during mass culture of POME-PD1. Apart from that, the final pH of culture medium was slightly lower than the initial pH of culture medium as growth arose.

4.3.3 The effects of different salinities and type of reactors on the production of biomass and carotenoids of *Rhodovulum sulfidophilum* grown in POME (POME-PD1)

The production of bacterial biomass was not influenced by salinity of culture medium and type of reactor (Appendix XIIa). At 60 h, bacteria grown in 30 ppt salinity medium in ziplock bag had the highest mean DW of 1.67 g/L, while the bacteria grown

in 0 ppt salinity medium in Schott bottle had the lowest mean biomass of 1.42 g/L (Table 4.1c). Hence, this marine bacterium species was able to grow in a broad range of salinities as the postdoc testing clearly demonstrated that biomass production was not significantly affected ($P > 0.05$) by type of reactor (ziplock bag, Schott bottle) and salinity (0, 5, 10, 20, 30 ppt).

On the other hand, production of bacterial carotenoids was greatly affected by type of reactor (Appendix XIIb). The total carotenoids of bacteria grown in ziplock bag were significantly higher ($P < 0.01$) than bacteria grown in Schott bottle irrespective of the salinity levels (Table 4.1c). In turn, the salinity did not differ ($P > 0.05$) with each other in terms of their total carotenoids.

4.3.4 The effects of different light intensities and type of reactors on the production of biomass and carotenoids of *Rhodovulum sulfidophilum* grown in POME (POME-PD1)

In the present study, light intensity had an effect on bacterial yield (Appendix XIIIa). The highest dry biomass was observed in cultures at a light intensity of 1.5 Klux, followed by 1.0 Klux, 2.5 Klux and lastly 3.0 Klux (Table 4.1d). At 60 h, the highest mean dry biomass of 14.28 g/L was obtained when bacteria was cultured in ziplock bags with a light intensity of 1.5 Klux (Figure 4.4), whereas the lowest mean dry biomass of 1.18 g/L was harvested from cultures in Schott bottle with a light intensity of 3.0 Klux (Table 4.1d). Hence, mean DW of biomass was significantly affected ($P < 0.01$) by light intensity.

Besides, biomass yields also influenced by type of reactor. Overall, bacteria grown in ziplock bag had significantly higher ($P < 0.05$) cell mass as compared to bacteria grown in Schott bottle (Table 4.1d). Nevertheless, posthoc testing shows no significant interaction effect ($P > 0.05$) among treatments in term of their biomass yield.

Figure 4.4 *Rhodovulum sulfidophilum* (PD1) grown in 25% POME (v/v) in ziplock bag with a light intensity of 1.5 Klux

Further, light intensity also had an effect on production of bacterial carotenoids (Appendix XIIIb). At 60 h, the highest mean total carotenoids was obtained from bacteria cultured in Schott bottle and given a light intensity of 2.5 Klux (1.45 mg/g), while the lowest was obtained from the one grown in ziplock bag and given a light intensity of 1.5 Klux (0.12 mg/g) (Table 4.1d). In brief, the mean total carotenoids of bacteria was only significantly influenced ($P < 0.04$) by light intensity but not by type of reactor ($P > 0.05$). Further, there was also no significant difference in light intensity and type of reactor interaction (Table 4.1d).

4.3.5 Calibration curve of *Rhodovulum sulfidophilum* grown in POME (POME-PD1)

Under the described conditions, the regression equation or calibration curve that was derived was:

$$\mathbf{CDW_{POME\text{-}PD1} = 0.8918\ OD_{660nm} + 0.2409}$$

Where,

$CDW_{POME\text{-}PD1}$ = cell dry weight of POME-PD1

OD_{660nm} = Optical density at 660 nm

0.2409 = the $CDW_{POME\text{-}PD1}$ intercept

This equation was used for estimating cell DW in future studies. At 60 h of cultivation, the maximum dry cell weight of bPOME-PD1 cultured in Schott bottle was 2.06 $\pm$ 0.69 g/L. The bacterium was cultured in 25% POME (v/v) at a pH of 4.55 and salinity of 30 ppt under a light intensity of 1.5 Klux.

4.4 Discussion

The present study has shown the significantly increased biomass production of POME-PD1 when cultured in ziplock bag. This bacterium was cultured under anaerobic-light condition to obtain high cell production (Noparatnaporn et al., 1983) and to maintain the predominant PB (Sawada & Rogers, 1977; Izu et al., 2001) as PB cultured in anaerobic environment rely on light as their energy source for photosynthesis. Pigmentation is also enhanced when grown in anaerobic-light condition when compared to aerobic-dark condition. In all bacterial experiments, tungsten lamp instead of fluorescent lamp was used. This is because tungsten lamp gives higher specific growth rate, biomass, carotenoid, cell yield, bacteriochlorophyll and protein concentration of bacteria than fluorescent lamp (Prasertsen et al., 1993) as it provides higher light intensity. Under tungsten lamp, bacteria cultures were able to gain more

energy to utilize the carbon sources in POME medium (Shaliza et al., 2006). However, the synthesis of photopigments might be depressed at higher light intensities and this indirectly reduces the photoheterotrophic growth of bacteria (Firsow & Drews, 1977).

The present study showed that PD1 cannot grow well in POME at concentrations higher than 50%. Poor growth has also been reported for B1 in undiluted sago effluent medium (Getha, 1995). However, B1 performs well in 25% effluent of sago starch processing (v/v in water) and even better in 50% effluent (v/v in water) (Getha et al., 1998a). The poor growth of PD1 in higher POME concentrations is likely due to limited transmission of light through the medium, attributable to the darker colour of POME as the concentration of POME increased. In all bacterial experiments, the pH of the culture medium decreased with incubation time. This could be caused by degraded products (Maheswari et al., 1998). The pH change in the medium can also be due to the co-relationship of sulfuric acid production with the assimilation or release of carbon dioxide (Pfennig, 1967; Sasaki & Nagai, 1979).

Bacteria growth and metabolism might be affected if they are grown in a medium of high alkalinity (pH 9 and above) because the nutrients such as copper, lead, chromium and zinc are completely precipitated out of the medium, except nickel and cadmium which are still present in low amounts (David et al., 1994). This is because bacteria require certain nutrients, in particular carbon, nitrogen, calcium, phosphorus, sodium, magnesium, iron, potassium, sulfur, manganese, zinc, cobalt, copper, molybdenum, selenium and nickel for good growth (Gerhard, 1986). However, in this study, PD1 cultured in pH 9 medium had comparable biomass with those grown in lower pH media (4.55 and 7.00) (see Table 4.1b). Hence, it is likely that PD1 still has the capability to grow and utilize the limited nutrients even with precipitation under high alkalinity since it has also been reported that PD1 is able to grow in pH 5.0 to 9.0 (Hansen & Veldkamp, 1973; Imhoff & Truper, 1992).

Salinity has also been reported to be crucial for marine PB as the right concentration of salinity will promote bacterial growth. For example, *Rhodovulum* sp. (PS88) had the highest growth when given 2% NaCl but did not grow in the absence of NaCl (Watanabe et al., 1998). The present study showed that POME-PD1 can also grow in the absence of NaCl. PD1 could tolerate salinity of up to 10% (Hansen & Veldkamp, 1973; Imhoff & Truper, 1992) but its optimal growth was observed at 2.5% NaCl (Imhoff, 2001). PB commonly require higher light intensities for optimal growth (Hillmer & Gest, 1977a; b). For example, the highest cell mass of 5.6 g/L (w/w) of *Rhodocyclus gelatinosus* was observed at 3.0 Klux as compared to 1.0 Klux when grown in seafood processing wastewater (Prasertsen et al., 1993). In the present study, PD1, however, grew well at a low light intensity (1.5 Klux) as compared to high light intensities (2.5 and 3.0 Klux) when grown in POME (see Table 4.1d). It has been reported that the carotenoid composition of bacteria is influenced by the culture medium and light intensity (Hirayama et al., 1974) as high light intensity suppresses carotenoid synthesis (Shipman et al., 1977). However, in the present study, high light intensity did not inhibit the production of carotenoid.

POME-PD1 grew better in ziplock bag as compared to Schott bottle. This was likely due to the thick glass material and low surface to volume ratio in the Schott bottle which limits the transmission of light through the culture medium. According to Richmond (2004), the light that activates photosynthesis can only penetrate ca. 3 cm into a culture medium. Further, the higher bacterial dry biomass obtained from POME-PD1 cultured in ziplock bag indicated that more carbon source had been utilized. It implies that PD1 is a potential bioremediator. A simple test of growing PD1 in 25% POME (v/v) for 60 h had shown 12% reduction in COD concentration. Although COD reduction is not fully investigated in this study, other workers had successfully reduced the COD of POME by using PB. For instance, 53% COD reduction was recorded after

96 h of incubation of B1 in 50% POME (v/v) (Shi, 2009), while 31.71% COD reduction was obtained after 66 h of incubation of B1 in 100% POME (v/v) (Zadariana, 2009).

In conclusion, mass culture and growth of POME-PD1 could be accelerated if the bacteria are grown in 25% POME in ziplock bags at a light intensity of 1.5 Klux. The growth of the bacteria, however was not affected by pH and salinity levels tested in this study.

CHAPTER 5

PHOTOTROPHIC BACTERIA GROWN IN PALM OIL MILL EFFLUENT AS A TOTAL FEED FOR THE ROTIFER, *BRACHIONUS ROTUNDIFORMIS*

Summary of significant findings

Brachionus rotundiformis, a planktonic rotifer, grew equally well on a complete diet of *Rhodovulum sulfidophilum* grown in palm oil mill effluent (POME-PD1), a phototrophic bacterium, as compared to *Nannochloropsis* sp., a microalga conventionally used in rotifer culture. Production, growth rate and fecundity of rotifers fed with unsettled culture of *R. sulfidophilum* grown in POME (cPOME-PD1) were superior to rotifers fed with settled biomass of *R. sulfidophilum* grown in POME (bPOME-PD1) under comparable food ration. The best performance in terms of the stated parameters were rotifers given 200 mL of cPOME-PD1 in 3 L of culture water, sustained at a mean population density of 300 to 600 ind/mL at Day 3 to 6. Rotifer populations given cPOME-PD1 as food, however, showed large variability in density, attributable to higher ammonia concentration from rotifer and bacteria excretion rather than POME. Although bPOME-PD1 and POME alone could support rotifer production, their combination (cPOME-PD1) enhanced rotifer production (maximum density of 898 ind/mL). The biochemical composition of rotifers fed with cPOME-PD1 was comparable to those fed with microalgae, except that rotifers fed with bacteria had significantly higher percentage of PUFA, especially DHA.

5.1 Introduction

In the present study, the main focus was to mass culture *Brachionus rotundiformis* in the shortest period as rotifers were required for feeding larval marble goby throughout the rearing period of 30 days. Newly hatched marble goby larvae with small mouth gape can only feed on them. Although *Artemia* nauplii were also used as

larval feed, it was only used as a co-feed starting from Day 20 to Day 30 of cultivation. Thus, their growth and reproduction are not studied in the present study. Commonly, newly hatched *Artemia* are directly fed to the fish larvae as they are the most nutritious at this stage and do not require to feed. Furthermore, *Artemia* cysts are easily available in market and cheap. Unlike *Artemia*, *Brachionus rotundiformis* cysts are not available in market, only *Brachionus plicatilis* cysts are available but their hatching rate is inconsistent. A steady supply of *Brachionus rotundiformis* is required as rotifers need to be fed once it hatches out.

The production of nutritionally rich rotifers and other live zooplankton for newly hatched fish larvae is a major requirement for successful larviculture. The major challenge is producing the live food in the shortest time and cost-effectively. Nonetheless, the success of the larviculture hatchery is highly dependent on the quality of the live microorganisms (James & Rezeq, 1989), and hence much effort has been made to improve the nutritional quality of rotifers particularly their protein, lipid and vitamin content (Coutteau & Sorgeloos, 1997). Rotifer's nutritive value is significantly correlated to its DW, biochemical composition, caloric level and ingested food (Lubzens et al., 1995). Although it is possible to produce large numbers of highly nutritious rotifers, this advantage may be offset by often poor production hygiene with cultures that may be contaminated by pathogens (Dhert et al., 2000). Besides the problem of producing quality rotifer feed in all aspects, the mass culture of rotifers is constrained by unpredictable harvests.

The use of PB as feed for rotifers has been largely unknown or unreported. Previous component studies (Chapter 3 and 4) had shown that rotifers fed on PB (see Table 3.2, page 52) and PB have the ability to metabolize POME (see Chapter 3 and Chapter 4). Hence, the use of PB to treat POME and its resulting biomass to feed rotifers will serve the dual purpose of bioremediation and aquaculture production. Thus,

the aim of this study was to investigate how rotifers can be mass produced from POME-PD1. POME-PD1 was selected as a rotifer feed because of its good nutritional profile (see Table 3.5, page 56; Figure 3.7, page 58). A batch culture production that reached a density of 600 rotifers/mL culture water (Lavens & Sorgeloos, 1996) or at least 50% of this density as harvestable can be potentially useful for larviculture of fish and invertebrates (Moksness et al., 2004).

5.2 Materials and methods

5.2.1 Production of *Rhodovulum sulfidophilum* grown in POME (POME-PD1)

One hundred millilitres of the phototrophic bacterium, PD1 cultured in 112 medium was aseptically inoculated into 900 mL of 25% POME (v/v). The bacteria were then cultured at 30 ± 2 ℃ under continuous illumination of 1.5 Klux with 100 watts tungsten bulbs (as previously determined; see Table 4.1d, page 69). The bacterial culture (liquid medium) was maintained anaerobically and the brownish-red bacteria were harvested at 60 h of cultivation (as previously determined; see Appendix Va-ii, page 208). The bacterial culture was either used directly as feed or was centrifuged at 2300 *g* for 20 min at 4 ℃ to sediment the biomass. The harvested biomass was then washed with 0.9% saline and the paste was kept at 4 ℃ until used.

5.2.2 Rotifer culture and condition

A consistent density of rotifers of seventy five ind/mL was used in all the experiments. Rotifer cultures were carried out in 4 L amber bottles with conical bottoms and working volume of 3 L of 5 ppt salinity water. Seawater was diluted to the required salinity by mixing with aged tap water before UV-irradiation. Fifty percent of culture water was sieved and replaced with new sterilized water daily. Throughout the rearing period, mild aeration was given and water quality parameters such as dissolved oxygen

(DO) concentration (mg/L), temperature (℃), pH, conductivity (mS) and salinity (ppt) were monitored daily using YSI 550A DO meter, YSI 100 pH meter and YSI EC300 conductivity meter. Ammoniacal-nitrogen (mg/L), nitrite (mg/L) and nitrate (mg/L) levels of the cultured water were measured at two-day intervals using HACH's 8155-salicylate method (Appendix XIVa), HACH's 8507-diazotization method (Appendix XIVb) and HACH's 8192-cadmium reduction method (Appendix XIVc), respectively (DR/2010 Spectrophotometer procedure manual, USA).

Rotifer numbers were estimated daily as number of ind/mL by first sampling 50 mL of rotifers from each culture tank and storing it in a pill box. The sampled rotifers were then killed by applying a drop of 40% (v/v) formaldehyde. In the laboratory, 1 mL of rotifers was sampled out from the pill box using a 1 mL Stempel pipette and then into a Sedgewick rafter cell before making the total count of rotifers under a microscope. The mean number of rotifers was calculated from three subsamples taken from each pill box.

The population growth rate (*r*) of rotifers was estimated based on the exponential growth equation: $\boldsymbol{r = (\ln N_t - \ln N_0)/t}$, where N_0 = initial population density and N_t = density of population after time t (days) (Krebs, 1985). The egg ratio was determined by dividing the number of amictic eggs by the number of rotifers.

5.2.3 Experimental designs

5.2.3.1 Experiment 1

The first experiment evaluated the effects of several forms of feed on rotifer production, growth and fecundity. The rotifer culture started with 75 ind/mL of culture water. Rotifers were fed three times daily with either POME-PD1 or marine microalgae *Nannochloropsis* sp., a common feed used for rotifer culture. The microalgal feed (Nanno 3600^{TM}) was commercially produced by Red Mariculture Inc., USA.

Triplicates of the following feed treatments were set up: Treatment 1a – settled bPOME-PD1; Treatment 1b – heat-killed bPOME-PD1; Treatment 1c – 60 h unsettled cPOME-PD1; Treatment 1d – microalgae.

Rotifers in all treatments received equal DW of feed. The DW of the bacteria and microalgae were predetermined prior to preparing the stock for feeding. The feed was, however, given in aqueous form, prepared by measuring the required wet weight of bacterial biomass (15 g) or microalgae (12 g) and suspending the particulates in 1 L of autoclaved 5 ppt salinity water to give an aqueous feed stock containing 1.68 g DW feed/L. For each tank of 3 L culture water, rotifers were fed a total daily ration of 100 mL of the aqueous feed stock or equivalent to approximately 0.168 g DW of feed biomass, given in three feeding portions: 30% in the morning, 30% in the afternoon and 40% in the evening. The given amount of feed was based on the preliminary success in culturing rotifers given one-tenth of the 1.68 g DW of biomass harvestable from 1 L of cPOME-PD1.

5.2.3.2 Experiment 2

The second experiment tested the effects of three food rations of bPOME-PD1 (prepared as aqueous feed stock as described above) and cPOME-PD1 (non-centrifuged) on rotifer production, growth and fecundity. The culture started with 75 rotifers/mL of culture water. The amount of feed given to rotifers was as described above. Rotifers were given the following total daily ration in three feeding portions: Treatment 2a – 100 mL of bPOME-PD1 feed stock; Treatment 2b – 200 mL of bPOME-PD1 feed stock; Treatment 2c – 300 mL of bPOME-PD1 feed stock; Treatment 2d – 100 mL of cPOME-PD1; Treatment 2e – 200 mL of cPOME-PD1; Treatment 2f – 300 mL of cPOME-PD1.

5.2.3.3 Experiment 3

The last experiment evaluated the separate and combined effects of POME and bacteria on rotifer production, growth and fecundity. All treatments used the best total daily ration of ca. 0.336 g organic matter (200 mL of feed stock) in three feeding portions. The treatments were as follows: Treatment 3a – 200 mL of cPOME-PD1; Treatment 3b – 200 mL of bPOME-PD1 feed stock; Treatment 3c – 200 mL of 25% diluted POME (no bacteria); and Treatment 3d – No feed (negative control). Each treatment was done in triplicates.

5.2.4 Fatty acid and amino acid analysis

Appropriate samples of rotifers were harvested at 96 h of cultivation. The harvested rotifers were those cultured in 5 ppt salinity. These samples were rinsed several times with filtered distilled water to discard salt. They were then immediately freeze-dried before fatty acid and amino acid analyses (Figure 5.1).

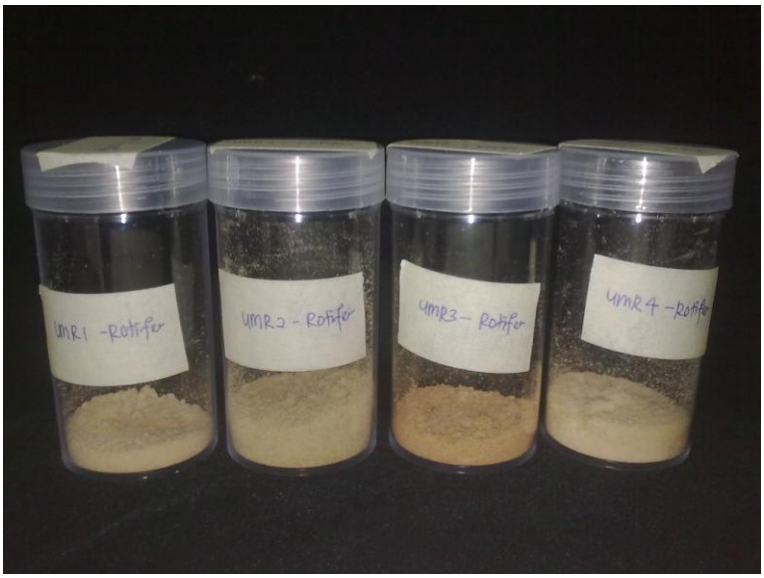


Figure 5.1 Freeze-dried rotifers

5.2.5 Statistical analysis

The highest rotifer density for each replicate of treatment regardless of day of culture was used for comparison. The actual data were subject to statistical analysis unless they did not fulfill the requirements of parametric testing and therefore, a $\log_{10}$ (X + 1) transformation (Zar, 1999) was applied. Only data from experiment 1 could not achieve normality and homogeneity of variance and hence, the Kruskal-Wallis test was used. Two-way ANOVA and posthoc Tukey HSD test were carried out on experiment 2 and 3 data. The highest daily growth rate and egg ratio among treatments were compared using one-way ANOVA (experiment 1) or two-way ANOVA (experiment 2 and 3). Statistical analysis was done using the computer software Statistica, version 9.

5.3 Results

5.3.1 Production of rotifers fed with bacterial diet compared to microalgal diet (Experiment 1)

The three forms of bacterial feed gave rotifer peak densities that were not significantly different from the microalgal feed (Kruskal-Wallis test, P = 0.183) (Appendix XVa). Nonetheless, the heat-killed bPOME-PD1 gave lower peak density (107 ind/mL) than the microalgae (212 ind/mL), bPOME-PD1 (221 ind/mL) and cPOME-PD1 (226 ind/ mL). All peak densities were reached either after the second or third day of culture (Figure 5.2).

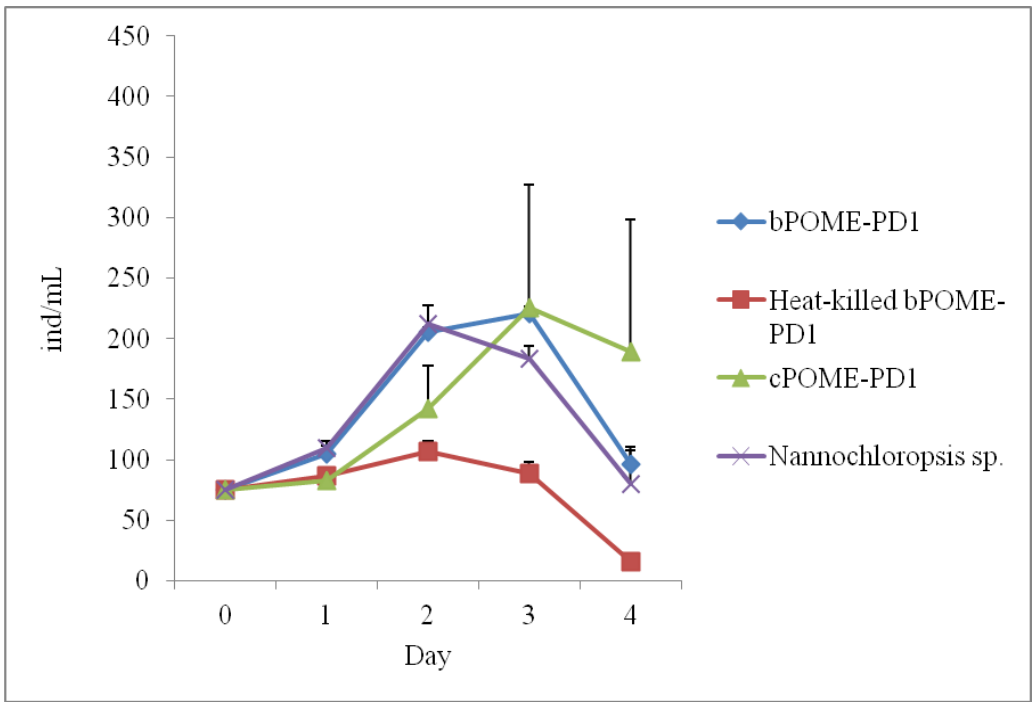


Figure 5.2 Production of rotifers (mean ± SE) reared for four days on different forms of bacterial diet and microalgal diet

The daily growth rate (*r*) of rotifers fed with bPOME-PD1 (at Day 1 or *r*1 = 0.33/d; at Day 2 or *r*2 = 0.67/d) was comparable with rotifers fed with microalgae (*r*1 = 0.38/d; *r*2 = 0.65/d), but higher than those fed with cPOME-PD1 (*r*1 = 0.10/d; *r*2 = 0.46/d) and heat-killed bPOME-PD1 (*r*1 = 0.14/d; *r*2 = 0.21/d) (Appendix XVb). However, rotifer populations either slowed down (bPOME-PD1 and cPOME-PD1) or crashed (microalgae and dried bPOME-PD1) after Day 2 (Table 5.1).

The egg ratio of rotifers fed with cPOME-PD1 varied within the range of 0.47 to 0.56 egg/rotifer throughout the 4-day rearing period and was significantly greater ($P < 0.01$) than other treatments (Table 5.1; Appendix XVc). The highest egg ratio was observed at Day 1 in all treatments, but egg ratio subsequently decreased, except in tanks given bacterial culture.

Table 5.1 Mean daily growth rate and egg ratio of rotifers fed with living (biomass of *Rhodovulum sulfidophilum* grown in POME - bPOME-PD1 and culture of *Rhodovulum sulfidophilum* grown in POME - cPOME-PD1) or non-living organisms (heat-killed bPOME-PD1 and microalgae) and cultured in 5 ppt salinity water

Day of culture	bPOME-PD1 (Treatment 1a)	Heat-killed bPOME-PD1 (Treatment 1b)	cPOME-PD1 (Treatment 1c)	*Nannochloropsis* sp. (Treatment 1d)
	Daily growth rate (r) (d^{-1}) (mean ± SD)[1]			
0 - 1	0.33 ± 0.11	0.14 ± 0.12	0.10 ± 0.11	0.38 ± 0.08
1 - 2	0.67 ± 0.15	0.21 ± 0.23	0.46 ± 0.36	0.65 ± 0.12
2 - 3	0.08 ± 0.05	-0.19 ± 0.12	0.32 ± 0.36	-0.15 ± 0.02
3 - 4	-0.86 ± 0.25	-1.70 ± 0.14	-0.40 ± 0.44	-1.01 ± 0.78
	Egg ratio (egg/rotifer) (mean ± SD)[1]			
1	0.46 ± 0.02	0.13 ± 0.03	0.54 ± 0.17	0.33 ± 0.01
2	0.13 ± 0.01	0.12 ± 0.03	0.47 ± 0.05	0.07 ± 0.01
3	0.06 ± 0.01	0.03 ± 0.00	0.52 ± 0.18	0.14 ± 0.01
4	0.08 ± 0.05	0.07 ± 0.10	0.56 ± 0.08	0.12 ± 0.05

[1] Mean of nine replicate values; see Appendix XV for detailed statistical test of significance

5.3.2 Production of rotifers fed with different rations of bacterial diet (Experiment 2)

Rotifer peak production was not significantly affected (ANOVA, $F = 2.750$, $P = 0.123$) by the type of bacterial feed (i.e. bPOME-PD1 or cPOME-PD1), but significantly affected by the ration or amount of bacterial feed given (ANOVA, $F = 19.782$, $P < 0.001$), with significant ration x feed interaction ($F = 20.794$, $P < 0.001$) (Appendix XVIa). Posthoc tests gave the following significant ($P < 0.05$) results for ration main effects: 200 mL (498 ind/mL) > 100 mL (323 ind/mL) > 300 mL (199 ind/mL), and ration x feed interaction effects: 200 mL of cPOME-PD1 (670 ind/mL) > 100 mL of cPOME-PD1 (380 ind/mL) = 300 mL of bPOME-PD1 (331 ind/mL) = 200 mL of bPOME-PD1 (325 ind/mL) = 100 mL of bPOME-PD1 (266 ind/mL) > 300 mL of cPOME-PD1 (66 ind/mL). The peak densities were achieved at a very variable number of days of culture (2 - 6 days) (Figure 5.3). In all treatments, rotifer populations fell or stagnated after three days of culture, except those given 200 mL of cPOME-PD1

(Figure 5.3). Those given 300 mL of cPOME-PD1, however, crashed from the start of the experiment.

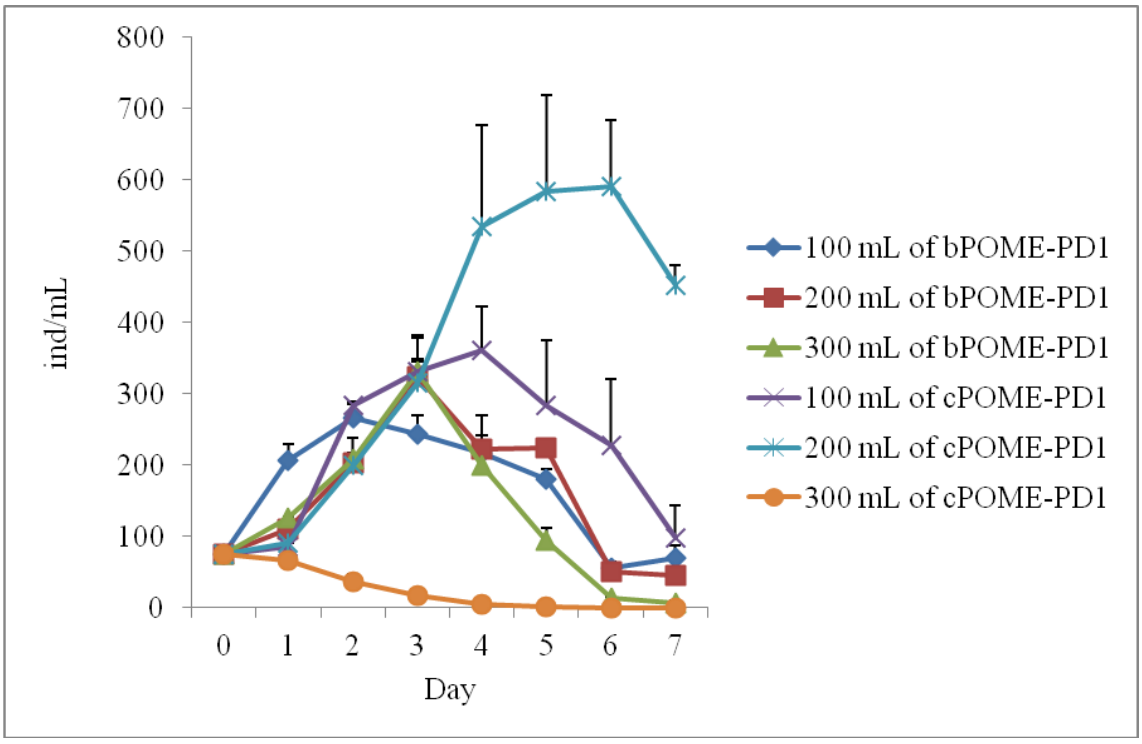


Figure 5.3 Production of rotifers (mean ± SE) reared for seven days on different rations of biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) and culture of *Rhodovulum sulfidophilum* grown in POME (cPOME-PD1)

As in the first experiment, the growth rates (*r* values) of the rotifer population in all treatments were generally the highest between one and two days of culture (Table 5.2). However, while populations given bPOME-PD1 immediately declined (negative *r*) after this or one day later, those given cPOME-PD1 (< 300 mL) were sustained longer (Appendix XVIb). In particular, 200 mL of cPOME-PD1 was able to sustain the rotifer population for up to 4 to 6 days and at an even higher peak density (Figure 5.3).

The rotifers fed with cPOME-PD1 had higher egg ratio than those fed with bPOME-PD1 regardless of food ration throughout the rearing period of 7 days (Appendix XVIc). The highest egg ratio was achieved at Day 1 by rotifers fed with 100

mL and 200 mL of bacterial culture (0.59 egg/rotifer). In all treatments, the egg ratio gradually decreased in magnitude after one day (Table 5.2).

Table 5.2 Mean daily growth rate and egg ratio of rotifers fed with different rations of bacterial food and cultured in 5 ppt salinity water

Day of culture	100 mL of bPOME-PD1 (Treatment 2a)	200 mL of bPOME-PD1 (Treatment 2b)	300 mL of bPOME-PD1 (Treatment 2c)	100 mL of cPOME-PD1 (Treatment 2d)	200 mL of cPOME-PD1 (Treatment 2e)	300 mL of cPOME-PD1 (Treatment 2f)
	Daily growth rate (r) (d^{-1}) (mean ± SD)[1]					
0 – 1	1.00±0.20	0.37±0.19	0.52±0.03	0.12±0.11	0.20±0.01	-0.13±0.19
1 – 2	0.26±0.07	0.62±0.15	0.51±0.06	1.21±0.13	0.73±0.36	-0.66±0.32
2 – 3	-0.09±0.06	0.47±0.08	0.44±0.22	0.16±0.10	0.46±0.05	-0.78±0.28
3 – 4	-0.11±0.13	-0.38±0.06	-0.67±0.51	0.06±0.23	0.48±0.21	-1.38±0.53
4 – 5	-0.19±0.07	0.02±0.14	-0.60±1.01	-0.36±0.45	0.12±0.22	-0.63±0.42
5 – 6	-1.19±0.41	-1.59±0.48	-1.90±0.29	-0.40±0.43	0.04±0.16	-
6 - 7	0.16±0.75	-0.04±0.27	-1.16±1.30	-0.90±0.28	-0.24±0.39	-
	Egg ratio (egg/rotifer) (mean ± SD)[1]					
1	0.12±0.01	0.26±0.04	0.28±0.03	0.59±0.03	0.59±0.09	0.19±0.15
2	0.06±0.03	0.16±0.01	0.20±0.04	0.25±0.04	0.42±0.11	0.25±0.17
3	0.03±0.00	0.06±0.01	0.05±0.01	0.13±0.04	0.43±0.15	0.06±0.02
4	0.02±0.00	0.08±0.01	0.11±0.01	0.12±0.03	0.25±0.12	0.21±0.11
5	0.07±0.03	0.09±0.03	0.04±0.00	0.13±0.06	0.27±0.08	0.07±0.06
6	0.23±0.05	0.10±0.07	0.12±0.16	0.16±0.09	0.24±0.19	-
7	0.02±0.01	0.00±0.00	0.17±0.15	0.10±0.05	0.22±0.12	-

[1] Mean of nine replicate values; see Appendix XVI for detailed statistical test of significance

5.3.3 The effects of bacteria and POME on rotifer production (Experiment 3)

The rotifer peak production was significantly affected ($P < 0.01$) by both bacteria and POME (200 mL), with the following main effects: With bacteria (611 ind/mL) > Without bacteria (328 ind/mL); With POME (715 ind/mL) > Without POME (223 ind/mL) (Appendix XVIIa). Nevertheless, the ANOVA indicated that 70.7% of the total variability in rotifer numbers could be explained by the presence of POME, while 24.7% was explained by the presence of the bacteria. Although the bacteria and POME interaction was not significant ($P = 0.170$), the rotifer population given both

bacteria and POME (i.e. cPOME-PD1) had the highest peak density (898 ind/mL), followed by POME only (533 ind/mL), bPOME-PD1 only (323 ind/mL) and no bacteria no POME (123 ind/mL). All treatments given higher amounts of feed (200 mL) gave high *r* values, either at Day 1 (bPOME-PD1) or Day 2 (cPOME-PD1 and POME) (Figure 5.4; Table 5.3). Only in the presence of POME were rotifer populations sustained up to the fourth day of culture, while rotifers fed on bacteria alone increased in numbers up to the third day before declining rapidly. However, the growth rate of rotifers was not significantly affected ($P > 0.05$) by both bacteria and POME (Appendix XVIIb).

The egg ratio of rotifers fed with cPOME-PD1 was significantly higher than those fed with bPOME-PD1, POME or no feed throughout the experiment (Appendix XVIIc). The highest egg ratio occurred at Day 1 and decreased subsequently in all treatments (Table 5.3).

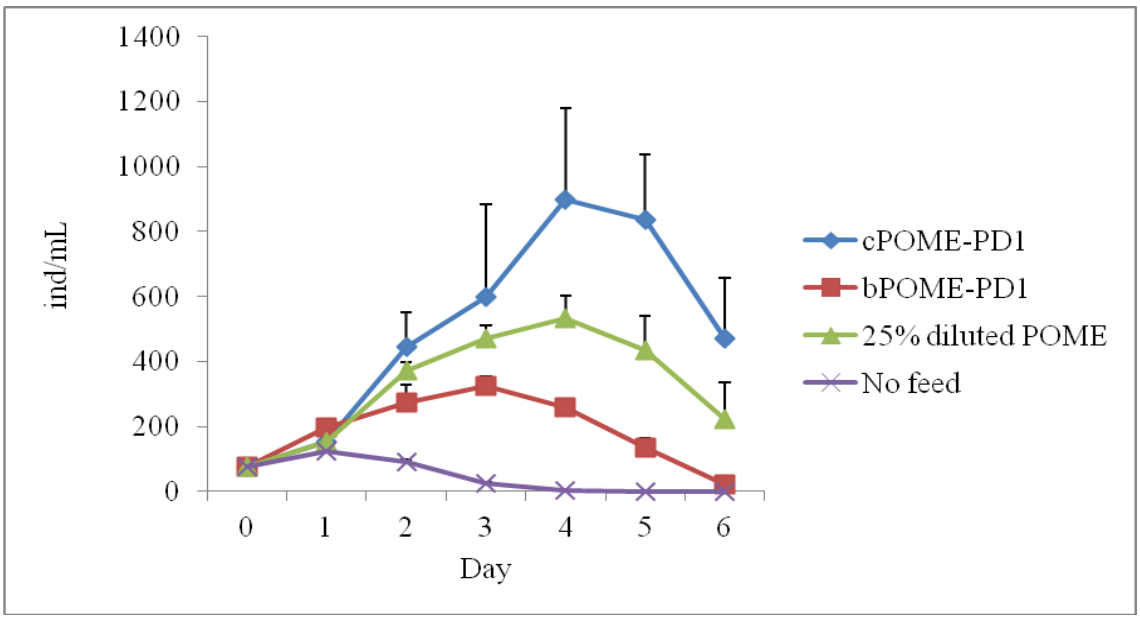


Figure 5.4 Effects of bacteria and POME on rotifer production (mean ± SE)

Table 5.3 Mean daily growth rate and egg ratio of rotifers fed with culture of *Rhodovulum sulfidophilum* grown in POME (cPOME-PD1) or biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) or POME or no feed and cultured in 5 ppt salinity water

Day of culture	200 mL of cPOME-PD1 (Treatment 3a)	200 mL of bPOME-PD1 (Treatment 3b)	200 mL of 25% POME (Treatment 3c)	No feed (Treatment 3d)
	Daily growth rate (r) (d^{-1}) (mean ± SD)[1]			
0 – 1	0.71±0.12	0.96±0.01	0.70±0.07	0.49±0.07
1 – 2	0.99±0.40	0.30±0.36	0.90±0.20	-0.30±0.20
2 – 3	0.13±0.50	0.19±0.27	0.24±0.04	-1.28±0.37
3 – 4	0.56±0.42	-0.21±0.15	0.11±0.09	-3.00±0.27
4 – 5	-0.03±0.23	-0.68±0.38	-0.26±0.27	-
5 - 6	-0.78±0.79	-2.57±1.64	-2.12±2.85	-
	Egg ratio (egg/rotifer) (mean ± SD)[1]			
1	0.56±0.09	0.27±0.03	0.29±0.02	0.02±0.01
2	0.33±0.08	0.26±0.09	0.22±0.02	0.01±0.00
3	0.32±0.11	0.25±0.02	0.17±0.04	0.01±0.01
4	0.27±0.16	0.05±0.02	0.14±0.04	0.00±0.00
5	0.15±0.09	0.09±0.02	0.11±0.04	-
6	0.19±0.09	0.22±0.20	0.13±0.11	-

[1] Mean of nine replicate values; see Appendix XVII for detailed statistical test of significance

5.3.4 Nutritional profile of freeze-dried rotifers

The percentages of protein, lipid, carbohydrate, ash and total amino acids were comparable among samples (Table 5.4). Rotifers fed with cPOME-PD1 recorded the highest PUFA (33.97%), followed by rotifers fed with microalgae (20.19%) and rotifers fed with bPOME-PD1 (17.87%). Rotifers fed with cPOME-PD1 had two-fold higher percentage of DHA and significantly lower EPA than rotifers fed with bPOME-PD1 or microalgae (Table 5.4; Appendix XVIIIa). Hence, their DHA/EPA ratio (1.72) was significantly much higher than the rest (Table 5.4). The amino acid compositions among rotifers fed with different feeds were comparable (Table 5.4; Appendix XVIIIb).

Table 5.4 Biochemical composition of freeze-dried rotifers cultured from different diet (mean of duplicate values)

Proximate profile	Rotifers fed with bPOME-PD1	Rotifers fed with cPOME-PD1	Rotifers fed with *Nannochloropsis* sp.
Proximate composition (%)			
Protein	47.6	60.0	56.0
Lipid	6.7	12.8	11.9
Carbohydrate	5.5	5.3	5.3
Ash	10.6	10.5	8.2
Moisture	29.6	11.4	18.6
Energy, Kcal/100g	272 (1142kJ)	376 (1579kJ)	352 (1479kJ)
Fatty acids (% total fatty acids)			
ARA	0.66	1.88	1.95
EPA	2.59	1.57	4.38
DHA	1.20	2.70	1.29
Total SFA	41.20	36.96	46.19
Total MUFA	40.93	29.07	33.62
Total PUFA	17.87	33.97	20.19
Percentage total fatty acids	100.00	100.00	100.00
n-3 PUFA	5.44	4.60	5.67
n-6 PUFA	12.02	24.62	13.14
ARA/EPA	0.26	1.20	0.45
DHA/EPA	0.46	1.72	0.29
DHA/ARA	1.81	1.44	0.66
Amino acids (% protein)			
Total essential amino acids	20.89	26.26	26.74
Total non-essential amino acids	23.58	27.99	28.81
Percentage total amino acids	44.47	54.25	55.55

See Appendix XVIII for detailed statistical test of significance

5.4 Discussion

5.4.1 The effects of living and non-living microorganisms on rotifer growth

Rotifers fed with live cultures (bPOME-PD1 or cPOME-PD1) had higher growth rates than those fed heat-killed bPOME-PD1 throughout the rearing period of 4 days, although statistical analysis showed no significant difference (Experiment 1). Non-viable organisms such as microalgae have been reported not to support rotifer growth (Baer & Goulden, 1998). The growth rates of rotifers fed with live bacteria were, however, comparable to those fed with commercial frozen *Nannochloropsis* sp. (see Table 5.1). The response of rotifers towards non-viable prey or food depends on their feeding habit. *Brachionus patulus*, an epiphytic form, normally consumes detritus and epiphytic algae (Ruttner-Kolisko, 1972), whereas *Brachionus calyciflorus* a strictly planktonic form consumes bacteria (Starkweather et al., 1979), planktonic algae and small ciliates (Gilbert & Jack, 1993). When these rotifers were given heat-killed *Chlorella* that settled to the bottom, *Brachionus patulus* showed higher population density than *Brachionus calyciflorus* (Ruttner-Kolisko, 1972). The present study supports this contention since *Brachionus rotundiformis* is strictly planktonic.

It has been reported that heat-killed bacteria may no longer be easily digestible and further lost their cytoplasmic content crucial to rotifer growth (Nguyen et al., 2005). Although the nutritional value of frozen algae has been demonstrated to be comparable to live algae (Lubzens et al., 1995), dried algae provide low rotifer growth as compared to live algae (Hirayama & Nakamura, 1976). It has been also suggested that rotifers performed better on live bacterial cultures attributable to their cell wall materials which enhanced rotifer digestive ability and metabolism (Nguyen et al., 2005). Bacteria can also produce vitamin B_{12} (Yu et al., 1988) and inorganic nutrients (Hessen & Andersen, 1990) which are essential elements for rotifer growth. Hence, the PB grown in POME are suitable as rotifer feed. The use of bacteria as an alternative rotifer feed can reduce

the reliance of microalgae for rotifer culture since continuous and successful mass culture of algae is known to be unpredictable, costly and labour intensive (Fukusho, 1983). The present study also showed that cPOME-PD1 was equally good as bPOME-PD1 for feeding rotifers. However, rotifers fed with cPOME-PD1 had higher variability in density as compared to other treatments (see Figure 5.2).

The second experiment showed significant difference between mean growth rates with respect to the different food rations given. Generally, rotifers fed with bPOME-PD1 or cPOME-PD1 increased in density with higher food ration until a limiting concentration of 300 mL or 0.504 g DW of bacterial food per 3 L of culture water (Treatment 2f). Presumably at this concentration of feed, there was no further increment in the ingestion rate of rotifer, and the high concentration of bacterial food could have a negative feedback on rotifer growth due to various factors including the excretory products from both rotifer and the bacteria (Montagnes et al., 2001). Rotifers fed with cPOME-PD1 could achieve higher population densities than those receiving bPOME-PD1 under comparable food ration, except at 300 mL of bacteria (see Figure 5.3). The higher rotifer density for those reared on cPOME-PD1 is likely due to the residual POME in it. POME is rich in lipids, carbohydrate, protein, nitrogenous compounds and minerals (Phang, 1990). Besides these nutrients, POME has been reported to contain a high amount of suspended organic matter (Ma, 2000), which could not be completely excluded despite the centrifuging and filtering process executed in the present study. In fact, it is possible to rear rotifers using only POME as food (Experiment 3). Nevertheless, densities of rotifers fed with POME or cPOME-PD1 were very variable (see Figure 5.4).

A mix of several phototrophic bacterial species could be considered to improve and stabilize total rotifer growth (Planas et al., 2004), although rapid growth leads to

lipid reduction as a result of DHA catabolism (Planas & Cunha, 1999). Thus, the benefits of PB grown in POME as rotifer feed needs further research.

5.4.2 The effects of feed on rotifer egg ratio

The zooplankton egg ratio is an important indicator for assessing the changes in their natural population (Razlutskij, 2000), nutritional state (Øie & Olsen, 1997), growth rate (Wolfinbarger, 1999) and general health (Onal et al., 2009). It has been shown that rotifer density is likely to increase regardless of the physicochemical parameters as long as the egg ratio is maintained above 40% (Onal et al., 2009).

The present study shows that rotifers fed with cPOME-PD1 had significantly higher egg ratio than other treatments. The egg ratio of rotifers given cPOME-PD1 ranged from 0.47 to 0.56 egg/rotifer which was consistent throughout the cultivation period (see Table 5.1). The egg ratio of bacteria-fed rotifers was superior (cPOME-PD1) to or as good (bPOME-PD1) as microalgae-fed rotifers (see Table 5.1), indicating that the bacterial feed had increased rotifer reproduction. Higher food rations of bacteria albeit to a limiting level further enhanced rotifer fecundity (see Table 5.2).

5.4.3 The effects of feed on water quality of rotifer culture

Rotifers excrete ammonia at levels depending on the type of food given (Kiorboe et al., 1985) and ca. 50% of the nitrogenous wastes produced by rotifer are ammonia (Hirata & Nagata, 1982). It has been reported that rotifers fed with *Nannochloropsis* sp. egest up to 84% of the ingested nitrogen into the culture medium as faeces (Hino et al., 1997). In the present study, tanks with rotifers fed with heat-killed bPOME-PD1 had relatively higher nitrite and nitrate but lower ammoniacal-nitrogen concentrations than those given living bPOME-PD1, cPOME-PD1 or microalgae (Experiment 1; Table 5.5). Therefore, the higher ammoniacal-nitrogen likely came from

both bacteria and rotifer excretion. Interestingly, the high ammoniacal-nitrogen level (1.05 – 5.07 mg/L) in rotifer cultures given bacterial feed (Treatment 1c) did not suppress rotifer growth although Dhert (1996) recommended a concentration of below 1 mg/L. However, Suantika et al. (2000b) pointed out that the negative effect could only be observed in batch culture of rotifers when ammoniacal-nitrogen level reached 10 mg/L. Yu and Hirayama (1986) reported a 50% decrease in rotifer growth and fecundity rates when the ammoniacal-nitrogen level reached 17.0 mg/L. Negative impacts due to undissociated ammonia could only be observed when the rotifer density reached 10^3 - 10^4 (Yoshimura et al., 1994). On the other hand, increasing the rate of water replacement positively improves water quality and indirectly stimulates rotifer production by removing the accumulation of wastes and stabilizing the culture medium (Fu et al., 1997).

Table 5.5 Water conditions (mean values) of the culture water of rotifers

Treatment	Day	Ammoniacal-nitrogen (mg/L)[1]	Nitrite (mg/L)[1]	Nitrate (mg/L)[1]	pH[1]	DO (mg/L)[1]	Conductivity (mS)[1]	Temperature (℃)[1]
1a	0	0.16	0.011	0.33	7.33	4.92	10.87	29.8
(100 mL of	1				7.11	4.26	10.05	30.1
bPOME-PD1)	2	1.41	0.188	0.44	7.10	4.28	9.96	29.8
	3				7.24	4.30	10.32	31.1
	4	3.47	0.381	0.99	7.22	4.45	9.87	29.6
1b	0	0.11	0.019	0.33	7.38	5.05	10.87	29.6
(100 mL of	1				7.25	4.82	9.97	29.7
heat-killed	2	1.04	0.239	0.77	7.19	4.79	9.93	29.6
bPOME-PD1)	3				7.30	4.67	10.26	31.2
	4	1.97	0.442	1.12	7.04	4.82	9.77	29.1
1c	0	1.05	0.015	0.22	7.03	4.49	10.96	29.4
(100 mL of	1				6.40	0.20	13.32	29.7
cPOME-PD1)	2	1.84	0.001	0.01	6.45	0.33	14.72	29.8
	3				6.71	0.34	15.08	31.2
	4	5.07	-0.002	-0.01	6.50	0.22	10.65	29.1
1d	0	0.15	0.036	0.57	7.26	4.79	9.98	30.3
(100 mL of	1				6.79	3.29	10.51	31.1
Nannochloropsis	2	2.27	0.033	0.18	6.88	4.21	9.84	28.7
sp.)	3				6.94	3.21	10.03	30.1
	4	4.32	0.044	0.18	6.76	4.15	9.54	27.6

Table 5.5, continued

Treatment	Day	Ammoniacal-nitrogen (mg/L)[1]	Nitrite (mg/L)[1]	Nitrate (mg/L)[1]	pH[1]	DO (mg/L)[1]	Conductivity (mS)[1]	Temperature (℃)[1]
2a	0	0.05	0.009	0.33	7.30	4.71	11.15	28.5
(100 mL of	1				7.25	4.80	11.20	28.5
bPOME-PD1)	2	0.79	0.070	0.40	7.24	4.86	11.09	28.3
	3				7.26	4.79	11.10	28.4
	4	0.97	0.085	0.51	6.69	4.58	10.81	29.7
	5				6.41	1.78	11.90	29.9
	6	2.61	0.058	0.22	6.41	2.46	11.24	30.9
	7				6.51	1.43	10.97	27.7
2b	0	0.08	0.016	0.52	7.26	4.86	11.19	28.3
(200 mL of	1				7.20	4.65	11.24	28.3
bPOME-PD1)	2	0.85	0.074	0.61	7.25	4.86	11.18	28.4
	3				7.24	4.79	11.20	28.3
	4	3.04	0.100	0.48	6.53	2.01	11.23	26.6
	5				6.46	2.08	12.31	28.5
	6	2.08	0.120	0.29	6.56	1.65	12.24	28.8
	7				6.86	1.98	12.02	28.6
2c	0	0.11	0.019	0.52	7.21	4.81	11.18	28.4
(300 mL of	1				6.97	3.56	11.18	28.5
bPOME-PD1)	2	0.72	0.075	0.61	7.06	3.94	11.19	28.5
	3				7.08	4.10	11.20	28.5
	4	3.31	0.079	0.39	7.09	3.83	10.49	30.1
	5				7.11	4.62	9.83	29.7
	6	0.99	0.100	0.35	7.20	4.28	10.11	31.3
	7				7.20	4.79	9.59	29.2
2d	0	3.00	0.018	0.25	6.73	4.42	10.90	30.1
(100 mL of	1				6.57	2.39	11.83	30.4
cPOME-PD1)	2	1.47	0.004	0.02	6.43	2.46	11.14	30.9
	3				6.84	2.71	10.93	27.7
	4	3.47	0.003	0.02	6.91	2.46	11.25	26.4
	5				6.86	2.49	12.41	28.8
	6	4.57	0.000	0.01	6.92	2.37	12.30	29.3
	7				7.09	2.95	12.00	29.2
2e	0	1.00	0.023	0.23	6.49	3.92	11.80	29.9
(200 mL of	1				6.48	0.35	13.53	30.1
cPOME-PD1)	2	5.13	0.009	0.01	6.34	0.55	12.15	30.6
	3				6.71	0.55	12.26	27.7
	4	7.40	0.013	0.06	6.38	0.35	13.10	26.5
	5				6.81	0.44	14.81	28.9
	6	9.63	0.038	0.05	6.90	0.70	14.56	29.2
	7				7.14	0.64	14.41	29.0

Table 5.5, continued

Treatment	Day	Ammoniacal-nitrogen (mg/L)[1]	Nitrite (mg/L)[1]	Nitrate (mg/L)[1]	pH[1]	DO (mg/L)[1]	Conductivity (mS)[1]	Temperature (℃)[1]
2f	0	1.77	0.011	0.19	6.35	3.27	12.68	29.7
(300 mL of	1				5.91	0.15	15.70	29.9
cPOME-PD1)	2	0.77	0.010	0.02	6.17	0.19	13.47	30.7
	3				6.42	0.16	13.77	27.6
	4	1.33	0.011	0.02	6.26	0.17	14.95	26.4
	5				6.36	0.22	17.70	28.7
	6	7.20	0.002	-0.01	6.84	0.13	17.18	28.8
	7				7.23	0.51	16.98	28.7
3a	0	0.45	0.007	0.21	6.67	3.55	11.33	28.6
(200 mL of	1				6.56	0.95	13.54	30.0
cPOME-PD1)	2	1.47	-0.001	-0.01	6.98	2.14	13.32	27.2
	3				6.91	1.63	13.74	28.6
	4	6.93	-0.002	-0.02	6.85	0.80	13.27	28.8
	5				6.75	0.42	15.58	31.1
	6	7.20	-0.002	-0.03	7.19	2.39	14.26	28.2
3b	0	0.43	0.010	0.29	7.35	4.90	9.45	28.3
(200 mL of	1				7.25	4.32	10.11	30.2
bPOME-PD1)	2	4.05	0.034	0.36	7.39	4.79	9.45	27.1
	3				7.29	4.14	9.70	28.4
	4	6.35	0.056	0.33	7.34	4.46	10.06	28.5
	5				7.19	3.75	10.52	31.2
	6	7.89	0.122	0.41	7.56	4.69	9.99	28.2
3c	0	0.26	0.003	0.20	6.72	4.60	11.18	28.3
(200 mL of	1				6.47	2.04	13.57	30.7
25% POME)	2	0.11	-0.009	-0.05	6.73	2.61	12.89	27.3
	3				6.54	1.21	13.69	28.8
	4	0.20	-0.007	-0.02	6.37	0.55	13.25	29.0
	5				6.12	0.22	15.67	31.6
	6	0.25	-0.010	-0.06	7.07	2.66	14.13	28.4
3d	0	0.11	0.008	0.57	7.69	5.51	9.87	28.7
(No feed)	1				7.93	6.08	9.56	30.6
	2	0.50	0.020	0.63	7.55	6.03	9.61	27.2
	3				7.62	6.12	9.78	28.4
	4	0.40	0.032	1.13	7.53	5.83	10.01	28.8

[1] Mean of triplicate values; negative value = undetectable

The present rotifer culture using cPOME-PD1 as feed had significantly lower levels of nitrite, nitrate and DO as compared to other treatments (Experiment 1; Table 5.5). The rotifers could survive in DO level as low as 0.20 mg/L for a few days even

though it was reported that in most rotifer cultures a minimum of 2 mg/L of DO is required (Lavens & Sorgeloos, 1996). The recorded temperatures in all culture tanks in the present study ranged from 28.7 to 31.2 ℃, indicating that temperature was not likely a limiting factor. In fact, *Brachionus rotundiformis* exhibited high growth and produced more resting eggs at higher temperature exceeding 25 ℃, but stopped reproducing eggs below 15 ℃ (Hirayama & Rumengan, 1993). The pH in the present study ranged from 6.45 to 7.38, which was also not limiting to rotifer growth since rotifers are naturally found in environments with pH levels of above 6.6 (Lavens & Sorgeloos, 1996). Increased rations of cPOME-PD1 but not bPOME-PD1 (Experiment 2; Table 5.5) significantly increased the ammoniacal-nitrogen in rotifer culture (Treatment 2d, 2e, 2f). This is because overfeeding could result in food being ingested but not digested (Galkovskaja, 1987) leading to deterioration of water quality (Dhert, 1996). Generally, tanks with rotifers fed with cPOME-PD1 had significantly higher ammoniacal-nitrogen but lower nitrite and nitrate levels than those given bPOME-PD1. Tanks with rotifers fed with POME alone (Experiment 3) had the lowest ammoniacal-nitrogen, nitrite and nitrate concentrations as compared to other treatments (Table 5.5). Therefore, POME or residual POME as present in the cPOME-PD1 had no or little effect on the ammonia level in the rotifer culture. Ammonia generation likely came from the excretion of bacteria and rotifers.

5.4.4 Rotifer biochemical composition

Information on the nutritive value of enriched rotifer diet is useful since it influences the growth performance of fish larvae. The protein content of rotifers fed with PB grown in POME is on the higher side given that the protein content of rotifers has been quoted to range from 28 to 67% of DW and appearing quite independent of a variety of diets (Øie & Olsen, 1997). Rotifers fed with cPOME-PD1 are also superior to

rotifers fed with microalgae in terms of their ARA/EPA, DHA/EPA and DHA/ARA ratios (see Table 5.4). Hence, rotifers produced on a diet of PB, PD1, will have an enriched nutritional profile. The production of high quality rotifers minimizes labour costs as no further enrichment is required to improve the survival and growth rate of the cultured fish larvae (Suantika et al., 2000a).

5.4.5 Bioconversion ability of rotifer

The fatty acid analysis reveals that rotifer has limited ability to bioconvert LNA to EPA and DHA. For example, rotifers fed with *Nannochloropsis* sp. had higher DHA but lower EPA, whereas rotifers fed with bPOME-PD1 had lower amounts of EPA and DHA as compared to their diet (Figure 5.5). Further, the ratios of DHA/LNA, EPA/LNA and ARA/LA in rotifers fed with bPOME-PD1 or *Nannochloropsis* sp. were only slightly higher as compared to their diets (Table 5.6). No or limited ability of rotifer and *Artemia* to bioconvert PUFA to HUFA had also been reported by other workers (Lubzens et al., 1985a; Howell & Tzoumas, 1991). Therefore, it is recommended that the live feed be fed with the right EFA ratio of diet prior to feeding to secure higher larval fish survival and growth.

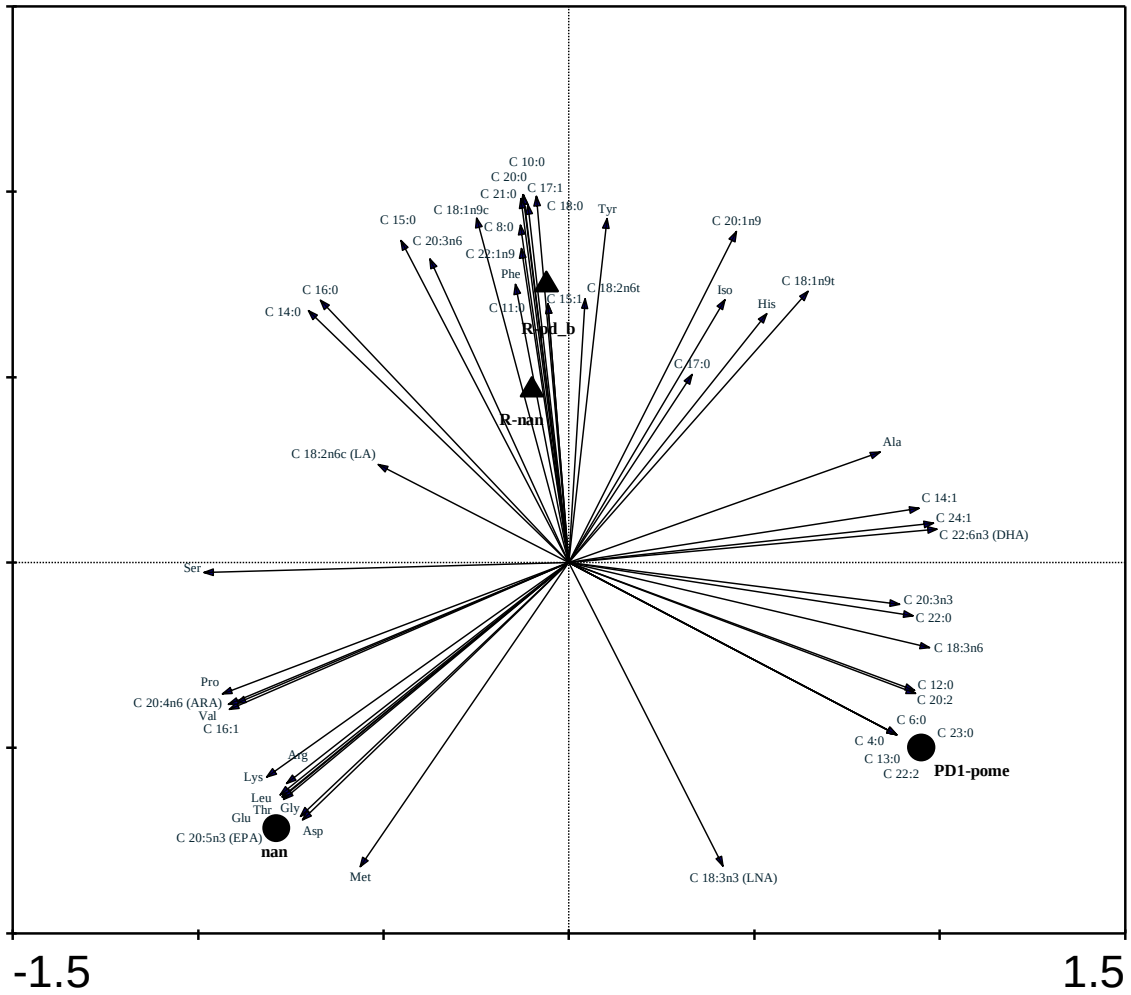


Figure 5.5 PCA of the fatty acid and amino acid compositions of freeze-dried phototrophic bacteria (PD1), microalgae (nan) and rotifers. Samples (filled circles, triangles): PD1-pome = bPOME-PD1; nan = *Nannochloropsis* sp.; R-pd_b = Rotifers fed with bPOME-PD1; R-nan = Rotifers fed with *Nannochloropsis* sp.; Variables (arrows): 37 fatty acids and 16 amino acids in conventional notations

Table 5.6 The ratios of DHA/LNA, EPA/LNA and ARA/LA of POME-grown phototrophic bacteria (PD1), microalgae (*Nannochloropsis* sp.) and rotifers

Organism	DHA/LNA	EPA/LNA	ARA/LA
bPOME-PD1	1.17	1.77	0.00
Nannochloropsis sp.	0.00	20.67	1.16
Rotifers fed with bPOME-PD1	1.88	4.05	0.14
Rotifers fed with *Nannochloropsis* sp.	-	-	2.41

In conclusion, PB grown in POME can support the batch production of rotifers in densities that are viable for larval fish culture. Although the use of cPOME-PD1 provides higher production of rotifers, this production is more variable as compared to

bPOME-PD1 which gives a lower but more stable rotifer density and better water quality. Rather the lower rotifer density from bPOME-PD1 appears to be due to rapid growth rate (*r*) that however falls rapidly due to high population numbers, lower food availability and self-regulation via reduced reproduction. In larviculture, the ability to produce good quality rotifers in high density and in the shortest time for feeding newly-hatched fish larvae is vital. Thus, the mass culture of rotifers that is entirely based on PB is recommended. The production of PB is simple, reliable and cheap, and can be completely sustained by POME, a discarded agroindustrial by-product.

CHAPTER 6

PHOTOTROPHIC BACTERIA GROWN IN PALM OIL MILL EFFLUENT AS A FEED FOR THE LARVAL MARBLE GOBY, *OXYELEOTRIS MARMORATA* (BLEEKER)

Summary of significant findings

The present study reports the significantly improved survival of marble goby larvae from previous reported studies (Senoo et al., 1994a; Senoo et al., 2008). Survival of marble goby larvae fed with biomass of *Rhodovulum sulfidophilum* grown in palm oil mill effluent (bPOME-PD1), microalgae (*Nannochloropsis* sp.) and live feed (rotifers and *Artemia* nauplii) cultured from bPOME-PD1 or microalgae, were evaluated at two salinities. Larvae fed directly with bPOME-PD1 had 0 - 29.0% survival and mean daily growth rate of 0.16 mm total length (TL)/day, whereas those fed directly with microalgae suffered total mortality after 30 days of culture in 5 ppt salinity water. Larvae fed with rotifers (Day 1 - 30) and *Artemia* nauplii (Day 21 - 30) both cultured from bPOME-PD1 in 5 ppt salinity, however, showed improved survival of 42.5 - 51.6% with a mean daily growth rate of 0.19 mm TL/day. In all experiments, fish larvae reared in 5 ppt salinity water showed significantly higher ($P < 0.01$) mean survival than those reared in 10 ppt salinity water. The survival of larvae fed the bacterial-based diet was higher compared to microalgal diet used in previous studies. The bPOME-PD1 had higher total PUFA and DHA than the microalgae which had very high EPA. However, larvae with very high ratios of DHA/EPA (> 11) or/and ARA/EPA (> 5), attributable to the given diet, suffered the highest mortality.

6.1 Introduction

Although the marble goby is a freshwater species, its larvae can survive in saline water to as high as 30 ppt salinity (Senoo et al., 2008). Nonetheless, high salinities (> 20

ppt salinity) gave higher larval mortalities as compared to lower salinities, with the best survival at 10 ppt (Senoo et al., 2008). It is unexpected that larvae reared in freshwater suffered high mortality of up to 100%, which was attributed to poor osmoregulation rather than food (rotifer) deprivation in fresh water (Senoo et al., 2008).

The requirement of dilute sea water for larval survival and growth of marble goby necessitates the use of salt tolerant prey food such as the euryhaline *Brachionus* spp. in order that uneaten live food could remain in the culture tank for a longer period. Although freshwater *Brachionus* spp. and phytoplankton have been used to feed larval marble goby (Tan & Lam, 1973; Senoo et al., 1994c), these planktons are difficult to mass produce under artificial conditions, as compared to the euryhaline *Brachionus* spp. where one species, *B. rotundiformis* has the added advantage of being small in size and hence, suitable for newly hatched marble goby larvae.

The present and conventional larviculture of marble goby is based mainly on the use of microalgae, such as marine *Nannochloropsis* sp. and *Chlorella* sp. which are fed directly or indirectly via zooplankton. Survival of the larvae is, however, very low. For instance, marble goby larvae given *Chlorella*-fed zooplankton had an average 10.1% survival over 70 days of culture (Senoo et al., 1994a), whereas those fed with *Nannochloropsis* sp. and cod oil-enriched zooplankton had 15.7% survival over 30 days of culture (Senoo et al., 2008). It has been pointed out that the common reliance on microalgae as zooplankton feed is not the best alternative for the future since most microalgae need very specific conditions to grow well (Michael, 1997).

Thus far, the use of PB in aquaculture has been at best supplementary to other fish feeds. Previous attempts to use PB as a complete diet for small larval fish have met with failure (Neik, 2006). One major reason is that PB grown in 112 medium has a poor content of lipids (Azad, 2004; see Table 3.5, page 56; Figure 3.7, page 58) which is vital to the early larval development of fishes whether as a source or precursor of HUFA

(John et al., 1999). Since POME is rich in lipids, the idea of using PB grown in POME as a direct feed for the small larvae of marble goby or via an intermediary consumer such as rotifer, appears promising and untested. Previous component study (Chapter 3) had demonstrated that marble goby larvae cultured in 10 ppt salinity and given live feed fed with bPOME-PD1 survived until the end of rearing period (see Table 3.4, page 54), while all died when given live feed fed with biomass of PB grown in 112 medium as food (see Table 3.3, page 53). Hence, the objective of the present study was to evaluate the effectiveness of using biomass of PB grown in POME as a direct or indirect (via rotifers and *Artemia* nauplii) live microbial feed for rearing marble goby larvae.

6.2 Materials and methods

6.2.1 Fish culture and condition

F1 generation marble goby adults were used as broodstock (Figure 6.1). A fish density of ten 1 dph marble goby larvae/L of water was used in all the experiments. Larval rearing and experiments were done in black, 150 L cylindrical tanks with conical bottoms (Figure 6.2) and working volume of 100 L of 5 ppt or 10 ppt salinity. The latter was previously tested to be the best among freshwater, 5 ppt, 10 ppt, 15 ppt, 20 ppt and 30 ppt salinity (Senoo et al., 2008). Throughout the rearing period of 30 days, 30% water replacement was done every 2-day intervals and water quality parameters such as DO concentration (mg/L), temperature (℃), pH, conductivity (mS) and salinity (g/L) were monitored daily, while ammoniacal-nitrogen (mg/L), nitrite (mg/L) and nitrate (mg/L) were measured every ten days. The live feed (rotifers and *Artemia* nauplii) density in the larvae culture was checked every day using a 1-mL Stempel pipette so as to maintain a density of 5 - 10 ind/mL.

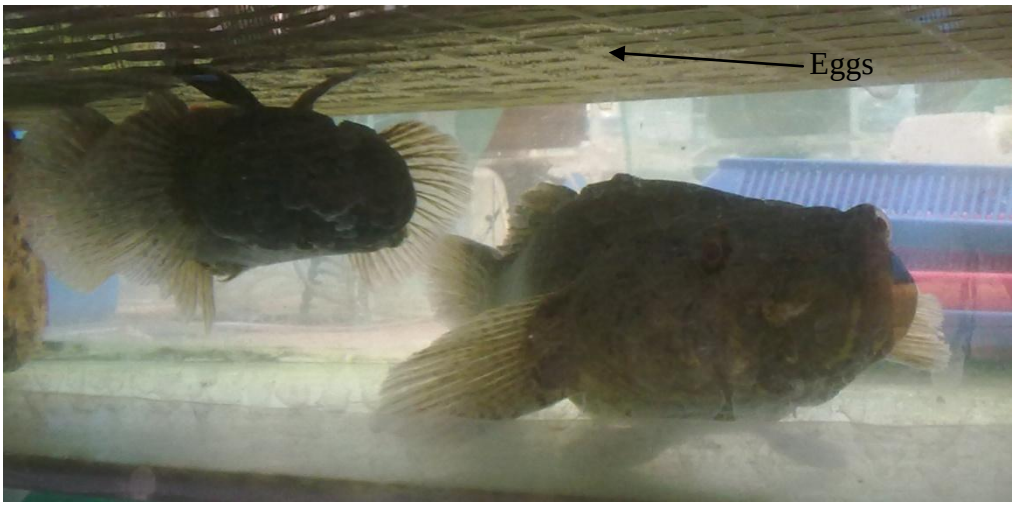


Figure 6.1 F1 generation marble goby adults

Figure 6.2 A cylindrical tank with conical bottom

6.2.2 Fish feeding

bPOME-PD1 or *Nannochloropsis* sp. were fed directly to fish larvae (Experimental 1) or indirectly via rotifers and *Artemia* nauplii (Experimental 2 and 3). Both bPOME-PD1 and *Nannochloropsis* sp. were fed directly to the larvae in aqueous form, prepared by measuring the required wet weight of bacterial biomass (15 g) or

microalgae (12 g) and suspending the cells in 1 L of autoclaved 5 ppt salinity water to give an aqueous feed stock containing 1.68 g dry weight feed/L. For each tank of 100 L culture water, fish larvae were fed a total daily ration of 400 mL of the aqueous feed stock equivalent to approximately 0.67 g DW of feed biomass.

The live feed were cultured in black, 150 L cylindrical tanks with conical bottoms and maintained in either 5 ppt or 10 ppt salinity. They were fed with either bPOME-PD1 or microalgae (*Nannochloropsis* sp.). The rotifers were first filtered through 200 µm- and then 40 µm-mesh nettings to discard the culture water and unwanted debris prior to larval feeding. bPOME-PD1 and microalgae were separately fed to 1 dph *Artemia* nauplii. After two days of feeding, the nauplii were harvested using a 150 µm-mesh netting. They were then rinsed twice with sterilized seawater before larval fish feeding. Rotifers were fed to larvae at the feeding rate of 10 ind/mL (Day 0 - Day 20) and 5 ind/mL (Day 21 - Day 30), while the bPOME-PD1 or microalgae-fed *Artemia* nauplii were given as additional feed from Day 21 onwards at a density of 5 ind/mL. Larval feeding experiments were only carried out for a period of 30 days, which represents the most critical phase of larval ontogeny (Tavarutmaneegul & Lin, 1988; Senoo et al., 2008).

6.2.3 Experimental designs

6.2.3.1 Experiment 1

The first experiment evaluated the efficacy of bPOME-PD1 to support larval fish survival and growth, compared to the microalgae *Nannochloropsis* sp. a common feed used for marble goby culture, in 5 ppt salinity and 10 ppt salinity at three periods of culture. The salinity, feed and time factors were crossed to give the following treatments: Treatment 1a - bPOME-PD1, 5 ppt salinity; Treatment 1b - *Nannochloropsis* sp., 5 ppt salinity; Treatment 1c - bPOME-PD1, 10 ppt salinity and Treatment 1d -

Nannochloropsis sp., 10 ppt salinity, which were tested for larval survival at Day 10, 20 and 30. One thousand 1 dph larvae were reared in triplicates tanks with a working volume of 100 L. Two feeding trials were carried out. In the first trial, all larvae died at the stocking density of 33 larvae/L. In subsequent trials, a stocking rate of 10 larvae/L was thus adopted.

6.2.3.2 Experiment 2

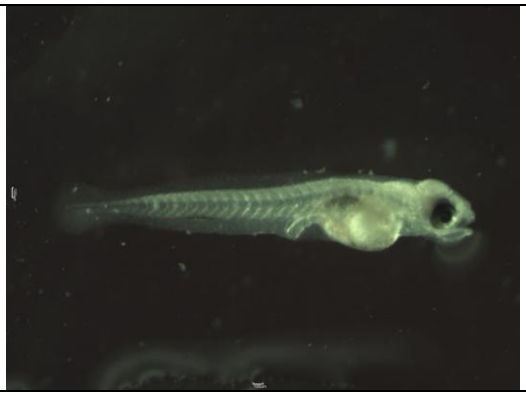
The second experiment tested the efficacy of live feed (rotifers and *Artemia* nauplii) fed with bPOME-PD1 on larval fish survival and growth in two salinities at the end of 30 days of culture. One thousand 1 dph larvae were reared in either 5 ppt salinity (Treatment 2a) or 10 ppt salinity (Treatment 2b) seawater with a working volume of 100 L with triplicates for each treatment. The larvae were fed twice daily with rotifers, and additionally, *Artemia* nauplii from Day 21.

6.2.3.3 Experiment 3

The final experiment evaluated the combined effects of salinity (5 ppt salinity or 10 ppt salinity) and feed type (live feed fed with bPOME-PD1 or live feed fed with *Nannochloropsis* sp.) and day of culture (Day 10, 20, 30) on larval fish survival and growth. The three factors were crossed to give the following treatments: Treatment 3a - live feed fed with bPOME-PD1, 5 ppt salinity; Treatment 3b - live feed fed with *Nannochloropsis* sp., 5 ppt salinity; Treatment 3c - live feed fed with bPOME-PD1, 10 ppt salinity; and Treatment 3d - live feed fed with *Nannochloropsis* sp., 10 ppt salinity, which were tested at Day 10, 20 and 30. All treatments included the bPOME-PD1 or *Nannochloropsis* sp.-fed *Artemia* nauplii from Day 21 onwards. Larvae were reared in triplicate tanks, each with a working volume of 100 L and stocked with one thousand 1 dph larvae.

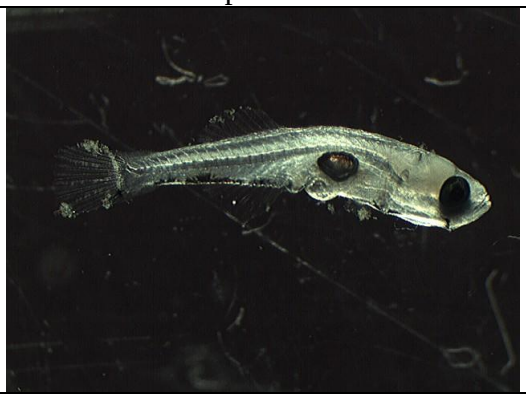
6.2.4 Survival and growth of marble goby larvae

The survival and growth of larvae were determined at 10-day intervals (Figure 6.3). Survival was determined by counting all the surviving larvae in the tank after completely draining off the culture water. After enumeration, the larvae were returned to their culture tanks with the same initial culture conditions. TL (mm) of three sacrificed larvae per tank were measured under a dissecting microscope (Leica MZ8) fitted to an imaging analysis system (Moticam 2500). Survival of fish larvae was measured in terms of percentage survival, while growth rate was measured in mmTL/day, over the period of consideration.

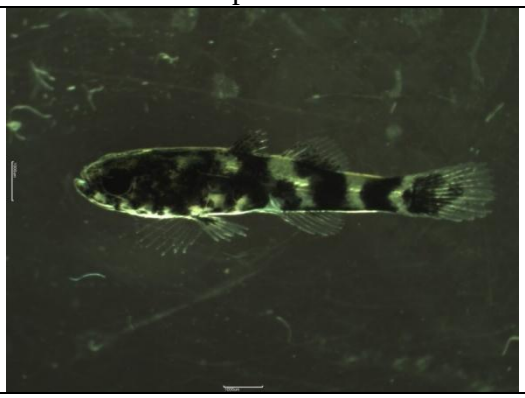


1 dph larvae

10 dph larvae



20 dph larvae



30 dph larvae

Figure 6.3 Early life stages of larval marble goby

6.2.5 Fatty acid and amino acid analysis

Appropriate samples of PB, rotifers and fish larvae were harvested at 48 h, 96 h and 15 d of cultivation, respectively. The harvested rotifers and fish larvae (Figure 6.4)

were those cultured in 5 ppt salinity which gave the best larval survival. These samples were rinsed several times with filtered distilled water to remove salt. They were then immediately freeze-dried before fatty acid and amino acid analyses (Figure 6.5).

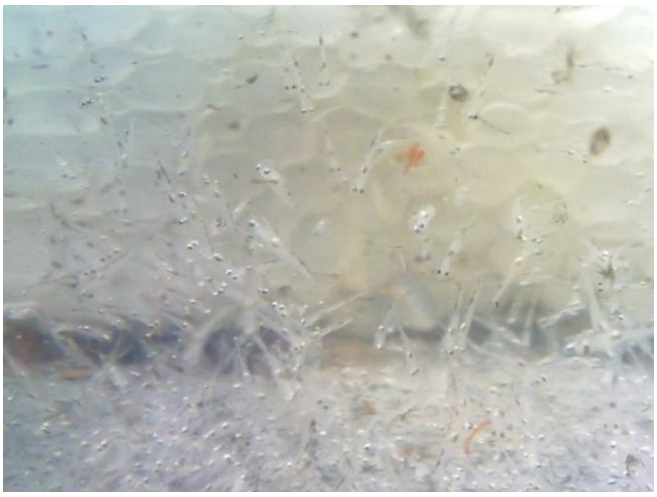


Figure 6.4 Fifteen days post-hatch (dph) marble goby larvae

Figure 6.5 Freeze-dried marble goby larvae

6.2.6 Statistical analysis

The mean survival and mean TL of larvae were calculated. For the first and third experiments, percent survival results were arcsine-transformed before parametric testing. Three-way ANOVA and posthoc Tukey HSD test were used to determine the main and

interaction effects of feed (bPOME-PD1, *Nannochloropsis* sp.), salinity (5 ppt, 10 ppt) and day of culture (Day 10, Day 20, Day 30) on larval survival and growth. For the second experiment, percent survival and TL of larvae at Day 30, cultured under two different salinities, were tested using the t-test. Statistical analysis was done using the computer software Statistica, version 9.

Principal components analysis (PCA) as a multivariate procedure was used to interpret the amino acid and fatty acid profiles of the PB, rotifers and larvae. In PCA, the profile data pertaining to *Nannochloropsis* sp. was based on the results reported by Red Mariculture Inc., USA (Anonymous, 2008). PCA was run on the software CANOCO 4.5 (Ter Braak & Smilauer, 2002).

6.3 Results

6.3.1 Survival and growth of larvae directly fed with either biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) or microalgae, in 5 ppt and 10 ppt salinity (Experiment 1)

In the first feeding trial where stocking density was 33 larvae/L, all larvae died. The microalgae-fed larvae in 10 ppt salinity died by the 10^{th} day of culture, whereas larvae in 5 ppt salinity died by the 20^{th} day. All larvae fed with bPOME-PD1 in both 5 ppt and 10 ppt salinity died by the 20^{th} day. In the second trial where the stocking density was 10 larvae/L, all microalgae-fed larvae in 10 ppt salinity died by the 10^{th} day, while all larvae in 5 ppt died by the 20^{th} day. However, larvae fed with bPOME-PD1 in 5 ppt salinity and 10 ppt salinity had 29.0% and 19.3% survival at the end of the feeding trial (30 days), respectively (Table 6.1).

The posthoc tests of the data obtained from the second trial showed that survival of larvae was significantly affected ($P < 0.01$) by the following main effects: feed type [bPOME-PD1 (41.1%) > microalgae (9.0%)], salinity [5 ppt (32.2%) > 10 ppt (17.9%)]

and day of culture [Day 10 (41.0%) > Day 20 (22.1%) = Day 30 (12.1%)] (Appendix XIXa). Significant interaction effect ($P < 0.01$) of salinity and day of culture on larval survival was only observed at Day 10 when the survival of larvae in 5 ppt salinity (56.9%) was significantly higher ($P < 0.01$) than in 10 ppt (25.2%). There was also significant ($P < 0.01$) interaction effect of salinity, feed and day of culture; mean survival of larvae was higher for bPOME-PD1 compared to microalgal feed irrespective of salinity, while larvae given microalgae survived better in 5 ppt than 10 ppt salinity at the Day 10 only. At the final day of the trial (Day 30), the mean survival due to feed and salinity effect is ranked (descending order) as follows: Treatment 1a (29.0%) > Treatment 1c (19.3%) > Treatment 1b = Treatment 1d (0%) (Table 6.1). At Day 30, TL of larvae fed bPOME-PD1 in 5 ppt and 10 ppt salinity was not significantly different ($P > 0.05$) (Table 6.1; Appendix XIXb).

Table 6.1 Mean survival, mean TL and mean daily growth rate of marble goby larvae fed with biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) or *Nannochloropsis* sp., and cultured in 5 ppt or 10 ppt salinity water for 30 days. Initial stocking size in 100 L = 1000 larvae.

Day of culture	5 ppt salinity, bPOME-PD1 (Treatment 1a)	5 ppt salinity, *Nannochloropsis* sp. (Treatment 1b)	10 ppt salinity, bPOME-PD1 (Treatment 1c)	10 ppt salinity, *Nannochloropsis* sp. (Treatment 1d)
		% survival (from Day 0)[1]		
0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
10	59.6 ±7.3	54.2 ±26.0	50.4 ±16.6	0.0
20	50.7 ±6.9	0.0	37.7 ±17.9	0.0
30	29.0 ±4.7	0.0	19.3 ±7.8	0.0
		Mean TL of larvae (mean mm ± SD)[2]		
0	3.85 ±0.09	3.85 ±0.09	3.85 ±0.09	-
10	5.41 ±0.35	5.18 ±0.45	5.27 ±0.56	-
20	6.99 ±0.93	-	7.24 ±0.52	-
30	8.72 ±0.30	-	8.31 ±0.59	-
		Mean daily growth rate (mmTL/day ± SD)[2]		
0-10	0.16 ±0.04	0.13 ±0.05	0.14 ±0.06	-
10-20	0.16 ±0.12	-	0.20 ±0.09	-
20-30	0.17 ±0.07	-	0.11 ±0.01	-

[1] Mean of triplicate values; [2] Mean of nine replicate values; see Appendix XIX for detailed statistical test of significance

6.3.2 Survival and growth of larvae fed with live feed cultured using biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1), in 5 ppt and 10 ppt salinity (Experiment 2)

The mean survival of larvae at Day 30 was significantly higher ($P < 0.01$) in 5 ppt salinity (Treatment 2a, 51.6%) compared to 10 ppt salinity (Treatment 2b, 18.4%) (Table 6.2; Appendix XXa). The mean TL of larvae at Day 30 in 10 ppt salinity (8.61 mm) was significantly longer ($P < 0.01$) than in 5 ppt salinity (7.68 mm) (Table 6.2; Appendix XXb).

Table 6.2 Mean survival, mean TL and mean daily growth rate of marble goby larvae given live feed fed with biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) in 5 ppt or 10 ppt salinity water for 30 days. Initial stocking size in 100 L = 1000 larvae. *Artemia* nauplii only introduced from Day 21.

Day of culture	5 ppt salinity, (Treatment 2a)	10 ppt salinity, (Treatment 2b)
	% survival (from Day 0)[1]	
0	100.0 ±0.0	100.0 ±0.0
10	94.2 ±1.6	89.9 ±3.5
20	88.7 ±8.1	54.0 ±23.5
30	51.6 ±4.0	18.4 ±11.2
	Mean TL of larvae (mean mm ± SD)[2]	
0	3.84 ±0.09	3.84 ±0.09
10	5.44 ±0.56	5.27 ±0.34
20	6.05 ±0.22	5.57 ±0.35
30	7.68 ±0.18	8.61 ±0.17
	Mean daily growth rate (mmTL/day ± SD)[2]	
0-10	0.16 ±0.06	0.14 ±0.04
10-20	0.06 ±0.05	0.03 ±0.00
20-30	0.16 ±0.01	0.30 ±0.03

[1] Mean of triplicate values; [2] Mean of nine replicate values; see Appendix XX for detailed statistical test of significance

6.3.3 The effects of feed, salinity and day of culture on larval survival and growth (Experiment 3)

Larval survival was significantly affected ($P < 0.02$) by the main effects of feed [microalgae-fed rotifers (51.9%) > bPOME-PD1-fed rotifers (41.8%)], salinity [5 ppt

(49.9% > 10 ppt (43.8%)] and day of culture [Day 10 (54.1%) > Day 20 (44.2%) = Day 30 (42.2%)], but with no significant interaction effects ($P > 0.05$) (Appendix XXIa). The results indicate that 46% of the larvae died in the first ten days of culture, while only a further 10% and 2% died from 10 - 20 days and 20 - 30 days, respectively. The mean larval survival at Day 30 for the different feed treatments is ranked (descending order) as follows: Treatment 3b (46.8%) > Treatment 3d (45.4%) > Treatment 3a (42.5%) > Treatment 3c (34.3%) (Table 6.3).

Total length of larvae was also significantly affected ($P < 0.01$) by feed [bPOME-PD1-fed rotifers (8.05 mm) > microalgae-fed rotifers (7.49 mm)] and day of culture [Day 30 (10.69 mm) > Day 20 (7.49 mm) > Day 10 (5.14 mm)] but not by salinity ($P > 0.05$) (Appendix XXIb).

Table 6.3 Mean survival, mean TL and mean daily growth rate of marble goby larvae given live feed fed with biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) or *Nannochloropsis* sp. in 5 ppt or 10 ppt salinity water for 30 days. Initial stocking size in 100 L = 1000 larvae. *Artemia* nauplii only introduced from Day 21.

Day of culture	5 ppt salinity, Live feed fed with bPOME-PD1 (Treatment 3a)	5 ppt salinity, Live feed fed with *Nannochloropsis* sp. (Treatment 3b)	10 ppt salinity, Live feed fed with bPOME-PD1 (Treatment 3c)	10 ppt salinity, Live feed fed with *Nannochloropsis* sp. (Treatment 3d)
		% survival (from Day 0)[1]		
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
10	54.3 ± 7.2	63.6 ± 26.1	40.0 ± 11.7	58.6 ± 6.3
20	43.2 ± 8.0	48.9 ± 3.8	36.4 ± 7.3	48.2 ± 7.0
30	42.5 ± 9.1	46.8 ± 2.9	34.3 ± 6.9	45.4 ± 6.6
		Mean TL of larvae (mean mm ± SD)[2]		
0	3.64 ± 0.12	3.64 ± 0.12	3.64 ± 0.12	3.64 ± 0.12
10	5.41 ± 0.64	4.93 ± 0.17	5.24 ± 0.18	4.97 ± 0.40
20	7.70 ± 0.41	7.31 ± 0.27	7.96 ± 0.19	6.97 ± 0.64
30	11.16 ± 1.05	10.77 ± 0.55	10.85 ± 0.34	9.97 ± 0.70
		Mean daily growth rate (mmTL/day ± SD)[2]		
0-10	0.18 ± 0.06	0.13 ± 0.03	0.16 ± 0.03	0.13 ± 0.05
10-20	0.23 ± 0.07	0.24 ± 0.04	0.27 ± 0.00	0.20 ± 0.05
20-30	0.35 ± 0.07	0.35 ± 0.04	0.29 ± 0.04	0.30 ± 0.04

[1] Mean of triplicate values; [2] Mean of nine replicate values; see Appendix XXI for detailed statistical test of significance

6.3.4 Water quality

The recorded water quality and environmental parameters among treatments and experiments were not distinctly different ($P > 0.05$). In all three experiments, the ammoniacal-nitrogen, nitrite and nitrate concentrations ranged from 0.08 to 0.36 mg/L, 0.22 to 1.25 mg/L and 0.011 to 0.495 mg/L respectively, while the water pH, DO, conductivity and temperature fell within the ranges of 7.18 to 7.92, 5.71 to 7.80 mg/L, 9.13 to 18.19 mS and 26.3 to 29.8 ℃ respectively (Table 6.4).

Table 6.4 Water conditions of the culture water of marble goby larvae

Day	Parameter[1]	Treatment 1a	Treatment 1b	Treatment 1c	Treatment 1d	Treatment 2a	Treatment 2b	Treatment 3a	Treatment 3b	Treatment 3c	Treatment 3d
0	Ammoniacal-nitrogen (mg/L)	0.08	0.08	0.10	0.09	0.11	0.12	0.23	0.26	0.27	0.21
	Nitrite (mg/L)	0.012	0.011	0.011	0.012	0.017	0.017	0.038	0.080	0.049	0.046
	Nitrate (mg/L)	0.26	0.27	0.30	0.29	0.26	0.22	0.49	0.74	0.49	0.51
	pH	7.41	7.44	7.51	7.51	7.74	7.92	7.68	7.57	7.41	7.62
	DO (mg/L)	6.80	6.77	6.80	6.79	6.16	6.37	6.85	6.89	6.94	6.96
	Conductivity (mS)	11.24	11.34	17.86	17.30	9.13	17.46	9.29	9.37	16.29	16.86
	Temperature (℃)	28.4	28.4	28.4	28.5	28.8	29.8	28.9	28.8	28.6	28.6
10	Ammoniacal-nitrogen (mg/L)	0.22	0.25	0.22	0.24	0.22	0.21	0.20	0.27	0.36	0.32
	Nitrite (mg/L)	0.013	0.030	0.025	0.027	0.055	0.042	0.186	0.047	0.106	0.050
	Nitrate (mg/L)	0.30	0.35	0.25	0.29	0.40	0.28	0.70	0.35	0.40	0.29
	pH	7.78	7.74	7.87	7.74	7.52	7.66	7.54	7.54	7.41	7.61
	DO (mg/L)	6.89	6.89	6.86	6.80	6.53	6.50	6.90	7.11	6.99	6.96
	Conductivity (mS)	10.94	9.78	17.45	18.12	9.36	17.15	9.50	9.47	16.92	17.55
	Temperature (℃)	27.8	27.8	27.8	27.8	26.3	26.5	29.1	29.1	29.0	29.2

Table 6.4, continued

Day	Parameter1	Treatment 1a	Treatment 1b	Treatment 1c	Treatment 1d	Treatment 2a	Treatment 2b	Treatment 3a	Treatment 3b	Treatment 3c	Treatment 3d
20	Ammoniacal-nitrogen (mg/L)	0.31	0.33	0.30	-	0.11	0.15	0.13	0.27	0.36	0.31
	Nitrite (mg/L)	0.026	0.041	0.036	-	0.050	0.054	0.342	0.157	0.495	0.156
	Nitrate (mg/L)	0.26	0.27	0.27	-	0.29	0.25	0.88	0.55	1.25	0.53
	pH	7.46	7.57	7.55	-	7.62	7.72	7.24	7.18	7.26	7.45
	DO (mg/L)	5.78	5.75	5.71	-	6.21	5.75	6.75	6.52	6.39	6.46
	Conductivity (mS)	10.96	9.61	17.44	-	9.83	18.11	9.79	11.44	16.44	18.19
	Temperature (℃)	28.1	28.1	28.1	-	28.6	28.5	29.6	29.2	29.2	29.3
30	Ammoniacal-nitrogen (mg/L)	0.19	-	0.14	-	0.20	0.19	0.08	0.13	0.16	0.14
	Nitrite (mg/L)	0.082	-	0.098	-	0.068	0.100	0.043	0.077	0.293	0.155
	Nitrate (mg/L)	0.46	-	0.36	-	0.36	0.31	0.35	0.48	1.08	0.63
	pH	7.28	-	7.43	-	7.50	7.64	7.56	7.54	7.31	7.46
	DO (mg/L)	6.15	-	5.85	-	7.31	7.42	7.46	7.54	7.43	7.80
	Conductivity (mS)	9.98	-	17.67	-	9.51	17.70	9.91	10.11	17.59	18.21
	Temperature (℃)	26.7	-	26.7	-	27.9	27.9	28.4	28.4	28.2	28.3

[1] Mean of triplicate values

6.3.5 Fatty acid and amino acid profiles of freeze-dried biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1), rotifers and marble goby larvae

In all the samples tested, the percentage of total SFA was higher compared to total MUFA and total PUFA. bPOME-PD1 had the highest total PUFA (42.83%) which is comparable to *Nannochloropsis* sp. (40.96%), while larvae given microalgae-fed rotifers had the lowest (8.30%) (Table 6.5). The total PUFA level of organism between trophic levels (i.e. from bPOME-PD1/microalgae to rotifer, or rotifer to fish) was reduced by approximately half, except for fish larvae given rotifers fed with bPOME-PD1. In the latter case, PUFA level in the larvae (24.59%) was slightly higher than its rotifer diet (17.87%) (Table 6.5).

The total percentage of non-essential amino acids was only slightly higher than essential amino acids in all samples except for bPOME-PD1 and fish larvae given rotifers fed with bPOME-PD1. Microalgal amino acids with a combined total of 98.13% were more than double bacterial amino acids (38.19%). The percentages of protein, lipid and carbohydrate in microalgae were also higher than in bPOME-PD1 (Table 6.5).

Biplots of the samples and the types of fatty acids and amino acids they contained were derived from PCA (Figure 6.6). The first and second PCA axis explained 47.9% and 40.0% of the total variation, respectively. The bPOME-PD1 contained significant amounts of certain SFA, MUFA and PUFA (particularly β-linolenic acid [C18:3n-6]; EPA and DHA), whereas the microalgae contained higher amounts of EPA and ARA. Both microorganisms, however, contained a fair amount of LNA. For amino acids, the bPOME-PD1 contained most of the amino acids but in lower amounts as compared to the microalgae. The former, however, contained higher amounts of histidine, isoleucine, alanine and tyrosine. Both consumers (rotifers and fish larvae) showed distinctly lower amounts of those amino acids and fatty acids found in

Table 6.5 Proximate composition (%), fatty acids (% total fatty acids) and amino acids (% protein) of freeze-dried phototrophic bacteria (PD1), microalgae, rotifers and marble goby larvae (mean of duplicate values)

Proximate profile	bPOME-PD1	*Nannochloropsis* sp.[1]	Rotifers fed with bPOME-PD1	Rotifers fed with *Nannochloropsis* sp.	Larvae fed with bPOME-PD1	Larvae fed with *Nannochloropsis* sp.	Larvae given rotifers fed with bPOME-PD1	Larvae given rotifers fed with *Nannochloropsis* sp.
Proximate composition (%)								
Protein	44.3	52.1	47.6	56.0	44.8	49.2	48.1	33.4
Lipid	7.4	16.7	6.7	11.9	6.4	5.2	5.7	5.6
Carbohydrate	7.4	16.0	5.5	5.3	6.7	9.7	9.1	18.6
Fatty acids								
ARA	0.00	3.94	0.66	1.95	3.60	4.11	0.10	0.44
EPA	3.94	31.42	2.59	4.38	0.57	0.79	2.11	0.14
DHA	2.60	0.00	1.20	1.29	6.88	8.81	8.35	0.37
Total SFA	45.42	21.34	41.20	46.19	57.13	55.41	49.94	68.75
Total MUFA	11.75	26.58	40.93	33.62	20.15	18.97	25.47	22.95
Total PUFA	42.83	40.96	17.87	20.19	22.72	25.62	24.59	8.30
Percentage total fatty acids	100.00	88.88	100.00	100.00	100.00	100.00	100.00	100.00
n-3 PUFA	10.73	32.94	5.44	5.67	8.10	10.33	14.51	0.51
n-6 PUFA	18.19	8.02	12.02	13.14	14.52	15.19	9.32	7.38
ARA/EPA	0.00	0.13	0.26	0.45	6.35	5.22	0.05	3.04
DHA/EPA	0.66	0.00	0.46	0.29	12.14	11.19	3.95	2.59
Amino acids								
Total essential amino acids	19.56	44.03	20.89	26.74	20.31	23.28	23.72	14.28
Total non-essential amino acids	18.63	54.10	23.58	28.81	22.40	23.39	23.63	15.12
Percentage total amino acids	38.19	98.13	44.47	55.55	42.71	46.67	47.35	29.40

[1] Red Mariculture Inc., USA; see Appendix IX, XVIII and XXII for detailed statistical test of significance

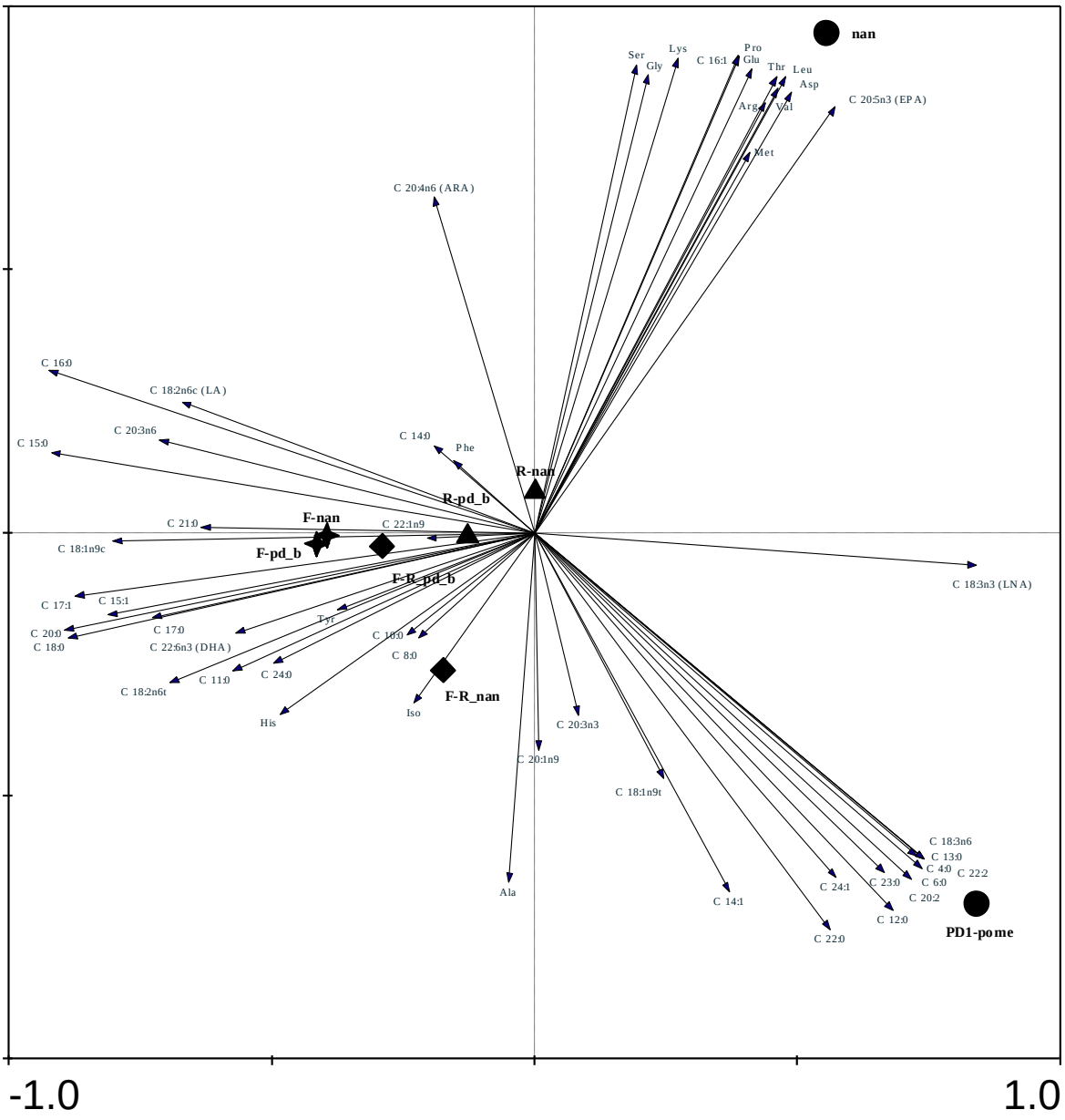


Figure 6.6 PCA of the fatty acid and amino acid compositions of freeze-dried phototrophic bacteria (PD1), microalgae (nan), rotifers and marble goby larvae. Samples (filled circles, diamonds, triangles, stars): PD1-pome = bPOME-PD1; nan = *Nannochloropsis* sp.; R-pd_b = Rotifers fed with bPOME-PD1; R-nan = Rotifers fed with *Nannochloropsis* sp.; F-pd_b = Fish larvae fed with bPOME-PD1; F-nan = Fish larvae fed with *Nannochloropsis* sp.; F-R_pd_b = Fish larvae given rotifers fed with bPOME-PD1; F-R_nan = Fish larvae given rotifers fed with *Nannochloropsis* sp.; Variables (arrows): 37 fatty acids and 16 amino acids in conventional notations

the microalgae. The larvae and their animal food (rotifers), however, did not differ greatly in both their profiles of fatty acid (Appendix XXIIa ; Appendix XVIIIa) and amino acid (Appendix XXIIb ; Appendix XVIIIb). Both fauna showed higher amounts of histidine, isoleucine, phenylalanine, tyrosine, DHA and LA as compared to the bPOME-PD1 and microalgae.

6.4 Discussion

6.4.1 Fish nutrition and survival

Significantly improved survival of marble goby larvae from previous studies (Senoo et al., 1994a; Senoo et al., 2008) has been demonstrated in the present study which shows the potential use of bPOME-PD1 as a complete diet for small larval fish. However, larval rearing is more successful via the larger sized live feed (rotifers and *Artemia* nauplii). Feeding marble goby larvae with a sole diet of bPOME-PD1 or microalgae (Experiment 1) appears difficult to sustain good survival, although bPOME-PD1 seems better than microalgae as food.

Larval marble goby suffers the highest mortality (up to 60%) during the first 10 days of ontogeny (see Table 6.1 – 6.3). High mortality (> 80%) at the early stage of development, especially from 4 - 9 days after hatching, has also been reported by others (Tan & Lam, 1973; Tavarutmaneegul & Lin, 1988). This may be due to poor feed nutrition or/and unsuitable prey size as the fish grows. Although most freshwater fishes do not strictly require long chain HUFA in their diet, they require LNA to manufacture *de novo* EPA and DHA (Sargent et al., 2002), which are regarded as EFA for good growth and neural development. Freshwater fishes, however, lack both the Δ12 desaturase enzyme which desaturates OA to LA, and Δ15 desaturase enzyme which acts to convert LA to LNA.

The higher percentage of DHA in marble goby larvae (6.88%) compared to its bPOME-PD1 diet (2.60%) indicates the fish's ability to bioconvert LNA in its diet to DHA. This likely conversion is also interpreted from the reduction in the amount of LNA in the bPOME-PD1 (2.22%) to much lower amounts in the fish (0.54%) (see Figure 6.6). Therefore, the high DHA/LNA (=12.74) ratio for larvae fed with bPOME-PD1 indicates the larva's bioconversion ability. Similarly, larvae fed microalgae also had a high ratio of 14.68. Larvae fed with rotifers on a bPOME-PD1 diet had an even higher ratio of 23.86. These results indicate that marble goby larvae are able to bioconvert LNA to DHA as long as LNA is provided in their diet. Nevertheless, marble goby larva has limited ability to bioconvert LA to ARA. This is evident from the ARA/LA ratio which only increased in larvae fed with bPOME-PD1 (compared to its diet). However, larvae fed with microalgae based diets or rotifers fed with bPOME-PD1 had lower ratio of ARA/LA as compared to their diets (Table 6.6). It appears that the marble goby larva lacks Δ5 and/or Δ6 desaturases to biosynthesize LA to β-LNA and finally to ARA. The use of mixed diet of live feed fed with POME-PD1 and POME-B1 could provide sufficient EFA to larvae as POME-B1 is rich in ARA and EPA, while POME-PD1 is rich in DHA (see Table 3.5, page 56). The mixed diet could further improve fish survival and growth, but this needs further feeding trials to substantiate.

Table 6.6 The ratios of DHA/LNA, EPA/LNA and ARA/LA of POME-grown phototrophic bacteria (PD1), microalgae (*Nannochloropsis* sp.), rotifers and marble goby larvae

Organism	DHA/LNA	EPA/LNA	ARA/LA
bPOME-PD1	1.17	1.77	0.00
Nannochloropsis sp.	0.00	20.67	1.16
Rotifers fed with bPOME-PD1	1.88	4.05	0.14
Rotifers fed with *Nannochloropsis* sp.	-	-	2.41
Marble goby fed with bPOME-PD1	12.74	1.06	0.59
Marble goby fed with *Nannochloropsis* sp.	14.68	1.32	0.65
Marble goby given rotifers fed with bPOME-PD1	23.86	6.03	0.02
Marble goby given rotifers fed with *Nannochloropsis* sp.	-	-	0.21

Given that both bPOME-PD1 and microalgae had high levels of LNA, the high mortality of marble goby larvae fed directly bPOME-PD1 or microalgae (see Table 6.1) could be the result of unsuitable DHA/EPA and ARA/EPA ratios in the larvae (see Table 6.5). Such ratios are good indicators of the fish's physiological status (Castell et al., 1994). Temperate marine fish larvae normally require DHA/EPA and ARA/EPA ratios of around 2.0 and 0.2 respectively to secure better growth and survival (Sargent et al., 1999a; b). On the other hand, higher DHA/EPA and ARA/EPA ratios have been reported in wild caught fry (2.9 - 8.8 and 0.3 - 1.6 respectively) and hatchery cultured fry (0.3 - 6.5 and 0.3 - 1.4) of three species of tropical marine fish (Ogata et al., 2004).

The few published works for freshwater fish also indicate lower DHA/EPA and ARA/EPA ratios in temperate waters as compared to tropical waters. For examples, values of respectively 0.2 - 0.8 and 0.39 - 0.5 were reported for the walleye (Sergiusz et al., 1999), and 1.5 and 1.0 for the Eurasian perch (Emilie et al., 2010). Wild-caught adult marble goby in Thailand had DHA/EPA and ARA/EPA ratios of 3.3 and 5.6 respectively, whereas the same ratios for cultured adults ranged from 2.9 - 8.0 and 2.0 - 4.5 respectively (Jatuporn, 2007). However, none of these values are higher than those reported in the present study. Thus, the very high ratios of DHA/EPA (> 11) or/and ARA/EPA (> 5), attributable to the given diet, are detrimental to the survival of marble goby larvae (see Table 6.1 and 6.5).

The optimum HUFA ratio for fish is species-specific and likely influenced by geography and ecosystem (Bell & Sargent, 2003). For instance, haddock larvae in temperate waters were found to incorporate higher ARA level and an optimum ratio of DHA:EPA:ARA of 40:5:4 was recommended (Castell et al., 2001). On the other hand, tropical marine fishes including those from mangroves were found to incorporate ARA at higher levels than cold water and temperate marine species (Ogata et al., 2006). The evidence thus far suggests that dietary ARA plays an important role in promoting

growth and survival of larval/juvenile marine fish compared to even diets with DHA only (Bell & Sargent, 2003). Interestingly, while increased ARA levels conferred larval sea bream increased ability to cope with stress due to handling, the larvae were more susceptible to stress induced by daily salinity fluctuations as a result of sustained ARA-mediated cortisol synthesis (Koven et al., 2001).

The bPOME-PD1 or microalgae severely lacked ARA or DHA, respectively. On the other hand, the microalgae had very high EPA. Hence, it is not clear why larvae fed with microalgae fared worse than those fed with bPOME-PD1 since both were 'biochemically' similar (see Table 6.5; Figure 6.6) and able to synthesize the HUFA they lacked. It may have to do with the extreme HUFA levels and the necessary physiological adjustments and accompanying stress the larvae had to undergo. This could explain why larvae (high ARA) cultured in higher salinity were more vulnerable than in lower salinity. Further study is however necessary to elucidate this.

Interestingly, the rotifers fed with microalgae or bPOME-PD1 were able to moderate the extreme levels of ARA, EPA and DHA present in their diet (see Table 6.5). Possibly due to this, fish larvae (lower DHA/EPA and ARA/EPA) fed with rotifers that were given microalgae or bPOME-PD1 survived better than those larvae fed with microalgae or bPOME-PD1. Hence, the introduction of rotifers as an intermediary between bPOME-PD1/microalgae and fish is beneficial.

As food size is known to affect larval survival (Konrad & Bardega, 1984), it is also possible that the size of the bacteria or microalgae had an influence on the survival of larval marble goby. The microscopic size of the bacteria (ca. 0.4 x 0.8 μm) or the microalgae (1 - 2 μm) is likely to constrain an optimal energy balance between the necessity for repeated prey captures (energy expenditure) and the assimilated food energy of the fish (Lavens & Sorgeloos, 1996). The present study shows that feeding

bPOME-PD1 to rotifers and then the latter to fish larvae not only provide adequate EFA and amino acids such as methionine (see Figure 6.6), but also a suitable prey size that likely incurs less energy expenditure. The slow moving, but not overly small rotifer is a suitable prey food because the marble goby larva is characteristically lethargic, has a small mouth gape (0.1 mm) and do not actively hunt its prey. This may be another reason why rearing of larvae was more successful when they were fed with live feed cultured from bPOME-PD1 as compared to larvae reared directly from bPOME-PD1. Nevertheless, PD1 is known to form bacteria flocs (Suzuki et al., 2009) which of suitable size may facilitate ingestion by fish larvae. The present study supports the conclusions of previous workers (Tavarutmaneegul & Lin, 1988; Senoo et al., 1994b) that suitable larval food (size and food quality) is crucial to the successful culture of marble goby larvae.

6.4.2 The effects of salinity and water quality

Marble goby larvae cultured in 5 ppt salinity demonstrated improved survival and growth rate over the previously reported optimum salinity of 10 ppt (Senoo et al., 2008). Although the marble goby is a freshwater fish, it could live equally well in brackish water such as in mangrove swamps and river mouths (Kottelat et al., 1993). Freshwater gobiid fishes came from the sea and entered freshwater a long time ago (Roberts, 1989), and many still exhibit an amphidromous life cycle (McDowall, 2007). The marble goby tolerates a wide range of salinity, to as high as 30 ppt (Chew et al., 2009). For this reason, the larvae of this species have been cultured in 10 ppt salinity at the nursing stage (Senoo, 2003; Senoo et al., 2008). The present study, however, shows that 10 ppt salinity was less favourable than 5 ppt salinity for the survival of marble goby larvae, for up to 30 days (see Table 6.1 - 6.3). Similarly, the larvae of the river

catfish *Pangasius sutchi* (Fowler) had a higher survival in 3 ppt than 12 ppt salinity, suffering complete mortality within two days in 12 ppt (Hardjamulia et al., 1988).

It is possible that marble goby larvae survived better in 5 ppt salinity because its diet contains a high salt content, e.g. bPOME-PD1 contained 3% (30 ppt salinity) salt. Thus, the larvae could have achieved a closer isomotic condition between body fluid and culture medium. Hence, the larval fish experiences less stress from osmoregulation or salt diffusion. It appears that in 10 ppt salinity, the larvae are not able to tolerate well the higher salt stress. On the other hand, in freshwater (0 ppt), the intake of salt-laden diet leads to the accumulation of higher ionic concentration of its body fluids leading to osmoregulatory stress or failure. Therefore, the inability of newly-hatched marble goby larvae to survive well in freshwater (Tan & Lam, 1973; Senoo et al., 1994b; Senoo et al., 2008) suggests their poor ability to osmoregulate and maintains the concentration of their body ions. The larval marble goby, however, minimizes its drinking rate and is able to avoid loss of body ions by producing highly diluted urine.

The mean daily growth rates of marble goby larvae obtained from the three experiments ranged from 0.03 to 0.35 mmTL/day (mean: 0.19 mmTL/day, n: 24) (see Table 6.1 - 6.3). The final mean TL of larvae (fed on either bPOME-PD1- or microalgae-fed live feed) cultured in 5 ppt and 10 ppt salinity showed little difference in growth rate, although maximum growth of brackishwater fish larvae has been hypothesised at an isosmotic condition of 10 ± 2 g/L which demands lower osmoregulatory energy (Brett, 1979). Nevertheless, fish growth also depends on the availability of energy and is influenced by both internal and external factors including salinity, stocking density and food quality (Jobling & Baardvik, 1994).

Water quality in the present study (see Table 6.4) fell within the recommended ranges given by Cheah et al. (1994), and there is no reason to suggest that poor water quality had affected larvae performance and hence obfuscated the results. Field data in

Boraphet reservoir, Thailand, showed that marble goby larvae thrive well in pH, temperature, DO of 6.5 to 7.2, 24 to 30 ℃ and 6.0 to 7.0 ppm respectively (Manop, 1984).

6.4.3 Advantages of using bacterial feed

bPOME-PD1 can be used as sole feed for rotifer production (see Figure 5.2, page 84; Figure 5.3, page 86 and Figure 5.4, page 88). Compared to microalgae, the cultivation of PB in POME is easier, faster and cheaper. It has been reported that some 30 to 40% (up to 70%) of a hatchery's operational costs are mainly used for microalgal culture. For instance, a specialised bivalve hatchery may spend USD 300 to 400/kg of dry algal biomass produced, and this cost would increase to USD 600/kg for smaller hatcheries (Coutteau & Sorgeloos, 1992). The high production cost of microalgae is mainly due to batch culture production, slow growth and low yields of microalgae, including the need for generally high technology and expertise (Michael, 1997).

6.4.4 Rearing marble goby larvae in clear water

To date, the fish larviculture industry is still dependent on microalgae as the main source of food for the newly-hatched larvae. For instance, the use of microalgae in green water technique is believed to enhance larval survival and growth as compared to clear water in fish culture. Microalgae influence larval nutrition (Howell, 1979; Scott & Middleton, 1979), behaviour (Naas et al., 1992), feeding (Reitan et al., 1993) and, microbiology (Nicolás et al., 1989) of the water. For example, although the protein conversion efficiency in larval turbot reared in clear water (18 - 28%) was higher as compared to those cultured in green water, the latter gave higher survival, initial growth, and appetite to larval turbot (Øie et al., 1997) than the former. Significantly higher amounts of trypsin and chymotrypsin had been found in marble goby larvae reared in

green water than those cultured in clear water (Van et al., 2005). These two proteolytic enzymes are important in enhancing food digestion leading to higher survival and growth of larval marble goby reared in green water as compared to clear water (Van et al., 2005). Nonetheless, in the present study, the larvae reared in clear water and given live feed such as rotifers and *Artemia* nauplii [both fed with microalgae or phototrophic bacteria (POME-PD1)] (see Table 6.2 – 6.3; Table 7.1 – 7.4, pages 134 – 135 & 137 – 138) had better survival and growth than those reared with microalgae (see Table 6.1).

In conclusion, the much improved survival of marble goby larvae based on bPOME-PD1 as feed, via rotifers and *Artemia* nauplii in 5 ppt salinity, provides an important breakthrough towards a cheap and stable mass production system for marble goby larvae. Thus, further investigations on the influence of stocking density and feeding regime on larval survival and growth are warranted.

CHAPTER 7

IMPROVING SURVIVAL AND MASS CULTURE OF MARBLE GOBY, *OXYELEOTRIS MARMORATA* (BLEEKER)

Summary of significant findings

Fish stocking density, type of feed and tank colour were evaluated on the survival and growth of marble goby larvae in 5 ppt salinity water. Fish larvae given live feed (rotifers and *Artemia* nauplii) fed with settled biomass of *Rhodovulum sulfidophilum* grown in palm oil mill effluent (bPOME-PD1) at a density of 15 larvae/L had significantly higher ($P < 0.01$) survival and longer TL (42.0%; 10.97 mm) than those at 20 larvae/L (33.5%; 9.74 mm) and 30 larvae/L (9.9%; 9.02 mm). Larvae reared on live feed fed with culture of *R. sulfidophilum* grown in POME (cPOME-PD1) or POME at a stocking density of 15 larvae/L, showed significantly improved ($P < 0.01$) survival (58.6 - 60.9%). The rearing of fish larvae in grey coloured tanks showed significantly improved ($P < 0.01$) survival and growth (79.0%; TL =11.44 mm) compared to larvae reared in black coloured tanks (32.5%; 10.91 mm) and transparent tanks (0.7%). Grey culture tanks, live feed fed with bPOME-PD1 or cPOME-PD1 and at the stocking density of 15 larvae/L support the best larval survival (71.4 - 81.9%) and growth (11.07 – 12.30 mm). Surviving larvae had low ratios of DHA/EPA (3 - 4) and ARA/EPA (0.1 – 2).

7.1 Introduction

Marble goby is one of the favoured freshwater fishes in Southeast Asia. Unfortunately, the large scale culture of this fish depends heavily on wild caught larvae that are becoming short of supply, while hatchery produced larvae are insufficient due to failure in establishing a stable culture technique. As a result, in Southeast Asia where it is mainly cultured, the current total production is only 684 tonnes (FAO, 2010).

Although there is considerable research on the hatchery production of larval marble goby (Tavarutmaneegul & Lin, 1988; Amornsakun et al., 2003; Senoo et al., 2008), none has reported on the use of POME or its products as fish feed. The previous component study (Chapter 6) had shown significantly improved survival and growth of larval marble goby compared to previous studies by using a diet based on only bPOME-PD1 (see Table 6.2, page 111; Table 6.3, page 112). The 30 dph larvae given live feed fed with bPOME-PD1 had significantly higher survival (42.5 - 51.6%) compared to the best survival using microalgae (ca. 10.0%) at a stocking density of 14 larvae/L and 5 ppt salinity (Senoo et al., 2008). This achievement is considered the first step towards establishing a stable and cheap culture technique for larval marble goby. Nevertheless, it may be possible to further improve larval survival for commercial production, by manipulating the culture conditions, such as fish stocking density, type of feed quality and tank colour.

The culture of marble goby larvae has used a stocking density of 14 larvae/L (Senoo et al., 2008) and 10 larvae/L (see Table 6.1 – 6.3), while high stocking density will potentially increase larval numbers, it has always resulted in high larval mortality and poor growth due to deterioration of water quality (Biswas et al., 2006) and larval stress (Tort et al., 1996). The optimal fish stocking density, however, varies with species, for instance, 30 larvae/L is favourable for turbot, but less than 10 larvae/L for halibut (Shields, 2001).

Previous studies had assessed the relative benefit of PB and POME to feed rotifers which are presumably of different nutritional quality; those fed with bPOME-PD1, POME, or cPOME-PD1 gave different peak densities due to the additive effects of PB and POME (see Figure 5.4, page 88). This feeding method could give different quality of live feed to larvae which in turn could affect larval survival and growth.

To date, there has been no report on the best tank colour for rearing larval marble goby. Marble goby larvae like most fish larvae are visual feeders, and their ability to survive when switching from endogenous feeding to exogenous feeding is likely dependent on easily detectable prey food (Ina et al., 1979; Blaxter, 1986). Conditions that optimize contrast between the environment and prey would enhance the detection and capture of food by larvae leading to good survival and growth (Downing & Litvak, 1999). Many studies have reported that fish and crustacean larvae survived well when cultured in black or dark coloured tanks (e.g. Blaxter, 1968; Howell, 1979; Matsuda et al., 1987; Martin-Robichaud & Peterson, 1998; Abed & Zeng, 2005; Jennifer & Stephen, 2009). Senoo et al. (2008) failed to obtain good survival and growth of marble goby larvae cultured in transparent tanks. The previous component study (Chapter 6) also shows that marble goby larvae survived well in black coloured tanks. Higher survival in the case of marble goby may be related to the fact that the marble goby naturally lives in a dark environment at the bottom of rivers, ponds and lakes, laying eggs at the bottom and is nocturnally active. Based on these facts, it is hypothesized that larval marble goby survive better in dark coloured tanks than in light coloured tanks. This hypothesis was tested in this study.

Hence, the objective of the present study was to investigate the effects of larval stocking density, tank colour and differentially-quality of live feed (rotifers and *Artemia* nauplii) on survival and growth of marble goby larvae.

7.2 Materials and methods

7.2.1 Fish culture and condition

Marble goby adults of F1 generation were used as broodstock. Larval rearing and experiments were conducted in either 30 L or 150 L cylindrical tanks with conical bottoms. The corresponding working volumes of culture water used were 25 L or 100 L

of 5 ppt salinity, respectively. This salinity was previously tested to be better than 10 ppt salinity for marble goby culture (see Table 6.1 – 6.3). Throughout the rearing period of 30 days, 30% water replacement was done every 2-day intervals and water quality parameters such as DO concentration (mg/L), temperature (℃), pH, conductivity (mS) and salinity (ppt) were monitored daily, whereas ammoniacal-nitrogen (mg/L), nitrite (mg/L) and nitrate (mg/L) concentrations were measured every 10-day intervals. The density of live feed (rotifers and *Artemia* nauplii) in the larvae culture was checked daily using a 1mL Stempel pipette in order to maintain a feeding density of 5 – 10 ind/mL.

7.2.2 Fish feeding

The live feed were cultured in black, 150 L cylindrical tanks with conical bottoms and maintained in 5 ppt salinity. They were fed with POME-PD1. The PB grown in POME were prepared in two forms for feeding; the biomass form (bPOME-PD1) which was obtained by centrifuging the cultured PB and the culture form (cPOME-PD1) which was the entire, unsettled, cultured broth of PB and residual POME.

The rotifers fed with POME-PD1 were first filtered through 200 µm- and then 40 µm-mesh nettings to discard the rotifer culture water and unwanted debris prior to larval feeding. Two-day old *Artemia* nauplii fed with similarly enriched bacteria were harvested using a 150 µm-mesh netting. They were then rinsed twice with sterilized seawater before larval feeding. The rotifers were fed to fish larvae at the feeding rate of 10 ind/mL (Day 0 - Day 20) and 5 ind/mL (Day 21 - Day 30), while *Artemia* nauplii were given to fish larvae from Day 21 onwards at a density of 5 ind/mL.

7.2.3 Experimental designs

7.2.3.1 Experiment 1

Since previous experiments had shown that marble goby larvae survived well at the stocking density of 10 larvae/L, whereas all died at 33 larvae/L (see Section 6.3.1, page 109), the present study evaluated the effects of three treatments of intermediate stocking densities, at 15, 20 and 30 larvae/L over three periods of culture (Day 10, 20, 30) on larval fish survival and growth. The two factors were crossed to give the following treatments: Treatment 1a - 1500 larvae; Treatment 1b - 2000 larvae and Treatment 1c - 3000 larvae, which were tested at Day 10, 20 and 30. Fish larvae were reared in 150 L black coloured tanks, each treatment in triplicate. The larval fish were fed twice daily with live feed cultured from bPOME-PD1.

7.2.3.2 Experiment 2

The second experiment evaluated the combined effects of feed quality (live feed fed with cPOME-PD1 or POME) and day of culture (Day 10, 20, 30) on survival and growth of fish larvae. The two factors were crossed to give the following treatments: Treatment 2a - live feed fed with cPOME-PD1 and Treatment 2b - live feed fed with POME, which were tested at Day 10, 20 and 30. One thousand and five hundred larvae (15 larvae/L) were stocked in 150 L black coloured tanks, in triplicates for each treatment.

7.2.3.3 Experiment 3

The third experiment determined the combined effects of tank colour (transparent, black and grey coloured tanks) and day of culture (Day 10, 20, 30) on larval fish survival and growth. The two factors were crossed to give the following treatments: Treatment 3a - transparent tank, Treatment 3b - black coloured tank and

Treatment 3c - grey coloured tank, which were tested at Day 10, 20 and 30. Three hundred and seventy-five larvae (15 larvae/L) were reared in 30 L tanks, in triplicates for each treatment. Larvae were given live feed fed with cPOME-PD1.

7.2.3.4 Experiment 4

The final experiment tested the combined effects of feed type (live feed fed with cPOME-PD1 or bPOME-PD1) and day of culture (Day 10, 20, 30) on survival and growth of larval fish. The two factors were crossed to give the following treatments: Treatment 4a - live feed fed with cPOME-PD1 and Treatment 4b - live feed fed with bPOME-PD1, which were tested at Day 10, 20 and 30. Three hundred and seventy-five larvae (15 larvae/L) were reared in 30 L grey tanks (Figure 7.1) in triplicates for treatment.

Figure 7.1 Thirty-litre cylindrical tanks with conical bottoms

7.2.4 Survival and growth of marble goby larvae

The survival and growth of fish larvae were determined at 10-day intervals. Survival of fish larvae was measured in terms of percentage survival, while daily growth rate was measured in mmTL/day, over the period of consideration.

7.2.5 Fatty acid and amino acid analysis

Appropriate samples of rotifers and fish larvae were harvested at 96 h and 15 d of cultivation, respectively. The harvested rotifers and fish larvae were those cultured in 5 ppt salinity. These samples were rinsed several times with filtered distilled water to remove salt. They were then immediately freeze-dried before fatty acid and amino acid analyses.

7.2.6 Statistical analysis

The mean survival and mean TL of fish larvae were calculated. For all the experiments, percent survival results were arcsine-transformed before parametric testing. Two-way ANOVA and posthoc Tukey HSD test were used to determine the main and interaction effects of two of the following factors: day of culture (Day 10, 20, 30), larval fish stocking density (15, 20, 30/L), type of feed (live feed fed with cPOME-PD1 or POME; live feed fed with cPOME-PD1 or bPOME-PD1) and tank colour (transparent, black, grey) on the survival and growth of fish larvae. Statistical analysis was done using Statistica, version 9.

Principal components analysis as a multivariate procedure was used to interpret the amino acid and fatty acid profiles of rotifers and fish larvae. PCA was run on CANOCO 4.5 (Ter Braak & Smilauer, 2002).

7.3 Results

7.3.1 The effect of fish stocking density on survival and growth of fish larvae given live feed fed with biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) (Experiment 1)

The main effects of fish stocking density [15 larvae/L (46.2%) = 20 larvae/L (44.0%) > 30 larvae/L (22.8%) and day of culture [Day 10 (53.4%) > Day 20 (31.3%) =

Day 30 (28.4%)] on larval fish survival were significant ($P < 0.01$), but with no significant interaction effect ($P > 0.05$) (Appendix XXIIIa). At Day 30, the mean survival at stocking density of 15 larvae/L (42.0%) was significantly higher ($P < 0.01$) than 20 larvae/L (33.5%) and 30 larvae/L (9.9%) (Table 7.1).

Total length of larval fish was significantly affected ($P < 0.01$) by the following main effects: stocking density [15 larvae/L (8.63 mm) > 20 larvae/L (7.86 mm) > 30 larvae/L (6.99 mm) and day of culture [Day 30 (9.91 mm) > Day 20 (7.94 mm) > Day 10 (5.63 mm)]. There was also significant ($P < 0.01$) interaction effect of stocking density and day of culture (Appendix XXIIIb). For instance, the mean TL of 30 dph fish larvae was significantly longer ($P < 0.01$) at a stocking density of 15 larvae/L (10.97 mm) compared to 20 larvae/L (9.74 mm) and 30 larvae/L (9.02 mm) (Table 7.1). Hence, a stocking density of 15 larvae/L was subsequently used for subsequent experiments.

Table 7.1 Mean survival, mean total length (TL) and mean daily growth rate of marble goby larvae given live feed fed with biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) in black tank for 30 days. Initial stocking size in 100 L = 1500 larvae or 2000 larvae or 3000 larvae. *Artemia* nauplii only introduced from Day 21.

Day of culture	15 larvae/L (Treatment 1a)	20 larvae/L (Treatment 1b)	30 larvae/L (Treatment 1c)
	% survival (from Day 0)[1]		
0	100.0 ± 0.00	100.0 ± 0.0	100.0 ± 0.0
10	53.8 ± 17.2	63.5 ± 8.9	43.0 ± 13.4
20	43.1 ± 11.7	35.0 ± 8.3	15.7 ± 9.7
30	42.0 ± 12.3	33.5 ± 8.5	9.9 ± 5.2
	Mean TL of larvae (mean mm ± SD)[2]		
0	3.77 ± 0.21	3.77 ± 0.21	3.77 ± 0.21
10	5.88 ± 0.22	5.61 ± 0.55	5.39 ± 0.32
20	9.04 ± 0.49	8.22 ± 0.59	6.55 ± 0.46
30	10.97 ± 0.43	9.74 ± 0.19	9.02 ± 0.49
	Mean daily growth rate (mmTL/day ± SD)[2]		
0-10	0.21 ± 0.02	0.18 ± 0.07	0.16 ± 0.05
10-20	0.32 ± 0.05	0.26 ± 0.03	0.12 ± 0.06
20-30	0.19 ± 0.05	0.15 ± 0.05	0.25 ± 0.06

[1] Mean of triplicate values; [2] Mean of nine replicate values; see Appendix XXIII for detailed statistical test of significance

7.3.2 The effect of type of feed (live feed fed with culture of *Rhodovulum sulfidophilum* grown in POME [cPOME-PD1] versus live feed fed with POME) on survival and growth of fish larvae (Experiment 2)

The mean survival of larval fish cultured in black tanks was significantly affected ($P < 0.01$) by the following main effects: type of feed [live feed fed with cPOME-PD1 (79.2%) > live feed fed with POME (69.9%)] and day of culture [Day 10 (90.9%) > Day 20 (72.9%) > Day 30 (59.7%)]. There was also significant ($P < 0.05$) interaction effect of type of feed and day of culture (Appendix XXIVa); the mean survival of 30 dph larvae given live feed fed with cPOME-PD1 (60.9%) was significantly higher ($P < 0.05$) than those given live feed fed with POME (58.6%) (Table 7.2).

Table 7.2 Mean survival, mean total length (TL) and mean daily growth rate of marble goby larvae given live feed fed with culture of *Rhodovulum sulfidophilum* grown in POME (cPOME-PD1) or POME in black tank for 30 days. Initial stocking size in 100 L = 1500 larvae. *Artemia* nauplii only introduced from Day 21.

Day of culture	Live feed fed with cPOME-PD1 (Treatment 2a)	Live feed fed with POME (Treatment 2b)
	% survival (from Day 0)[1]	
0	100.0 ±0.0	100.0 ±0.0
10	98.2 ±0.7	83.6 ±6.4
20	78.4 ±7.2	67.4 ±7.8
30	60.9 ±8.4	58.6 ±7.8
	Mean TL of larvae (mean mm ± SD)[2]	
0	4.00 ±0.10	4.00 ±0.10
10	5.60 ±0.38	5.19 ±0.10
20	8.68 ±0.37	8.51 ±0.39
30	9.74 ±0.50	9.91 ±0.57
	Mean daily growth rate (mmTL/day ± SD)[2]	
0-10	0.16 ±0.04	0.12 ±0.02
10-20	0.31 ±0.07	0.33 ±0.05
20-30	0.11 ±0.06	0.14 ±0.09

[1] Mean of triplicate values; [2] Mean of nine replicate values; see Appendix XXIV for detailed statistical test of significance

The mean TL of fish larvae was significantly increased ($P < 0.01$) with day of culture [Day 30 (9.83 mm) > Day 20 (8.59 mm) > Day 10 (5.39 mm)], but not affected by type of feed (Appendix XXIVb). The mean TL of 30 dph larvae given live feed fed with POME (9.91 mm) was not significantly ($P > 0.05$) different compared to those given live feed fed with cPOME-PD1 (9.74 mm) (Table 7.2).

7.3.3 The effect of tank colour on survival and growth of fish larvae (Experiment 3)

Larval survival was significantly affected ($P < 0.01$) by the following main effects: tank colour [grey (85.1%) > black (42.2%) > transparent (16.5%)] and day of culture [Day 10 (66.7%) > Day 20 (39.7%) = Day 30 (37.4%)]. There was also significant ($P < 0.01$) interaction effect of tank colour and day of culture (Appendix XXVa). For example, the mean survival of 30 dph larvae for tank colour is ranked as follows: grey (79.0%) > black (32.5%) > transparent (0.7%) (Table 7.3).

However, the mean TL of 30 dph larvae reared in black tank (10.91 mm) and grey tank (11.44 mm) was not significantly different ($P > 0.05$) (Table 7.3; Appendix XXVb). TL of 20 dph and 30 dph larvae cultured in transparent tanks were not measured due to few surviving larvae.

Table 7.3 Mean survival, mean total length (TL) and mean daily growth rate of marble goby larvae given live feed fed with culture of *Rhodovulum sulfidophilum* grown in POME (cPOME-PD1) in 5 ppt salinity water and reared in transparent tank, black tank or grey tank for 30 days. Initial stocking size in 25 L = 375 larvae. *Artemia* nauplii only introduced from Day 21.

Day of culture	Transparent tank (Treatment 3a)	Black tank (Treatment 3b)	Grey tank (Treatment 3c)
	% survival (from Day 0)[1]		
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
10	47.5 ± 5.5	58.8 ± 14.3	93.8 ± 6.5
20	1.2 ± 1.9	35.3 ± 4.7	82.6 ± 7.3
30	0.7 ± 1.2	32.5 ± 4.0	79.0 ± 10.2
	Mean TL of larvae (mean mm ± SD)[2]		
0	3.83 ± 0.15	3.83 ± 0.15	3.83 ± 0.15
10	4.52 ± 0.25	5.19 ± 0.04	5.19 ± 0.44
20	-	7.46 ± 0.97	8.61 ± 0.15
30	-	10.91 ± 0.64	11.44 ± 0.21
	Mean daily growth rate (mmTL/day ± SD)[2]		
0-10	0.07 ± 0.01	0.14 ± 0.02	0.14 ± 0.03
10-20	-	0.23 ± 0.09	0.34 ± 0.05
20-30	-	0.35 ± 0.16	0.28 ± 0.01

[1] Mean of triplicate values; [2] Mean of nine replicate values; see Appendix XXV for detailed statistical test of significance

7.3.4 The effect of type of feed (live feed fed with culture of *Rhodovulum sulfidophilum* grown in POME - cPOME-PD1 versus live feed fed with biomass of *Rhodovulum sulfidophilum* grown in POME - bPOME-PD1) on survival and growth of fish larvae (Experiment 4)

The prior experiments suggested that marble goby should survive best if cultured in grey tank and stocked at a density of 15 larvae/L. Thus, under these conditions, the final experiment evaluated which type of feed, i.e. live feed grown using cPOME-PD1 or bPOME-PD1, was better in term of fish survival.

The survival of larvae significantly decreased ($P < 0.05$) with day of culture [Day 10 (93.6%) > Day 20 (80.6%) > Day 30 (76.7%)], but was not significantly affected ($P > 0.05$) by type of feed (Appendix XXVIa). At the final day of the experiment (Day 30), the mean survival for fish larvae given live feed fed with cPOME-

PD1 (81.9%) was not significantly different (P > 0.05) compared to those given live feed fed with bPOME-PD1 (71.4%) (Table 7.4).

Total length of larval fish was significantly longer (P < 0.01) with day of culture [Day 30 (11.69 mm) > Day 20 (8.99 mm) > Day 10 (5.52 mm)], but was not affected by type of feed (P > 0.05). There was also significant (P < 0.01) interaction effect of type of feed and day of culture (Appendix XXVIb); mean TL of 30 dph fish larvae given live feed fed with bPOME-PD1 (12.30 mm) was significantly longer (P < 0.01) compared to those given live feed fed with cPOME-PD1 (11.07 mm) (Table 7.4).

Table 7.4 Mean survival, mean total length (TL) and mean daily growth rate of marble goby larvae given live feed fed with culture of *Rhodovulum sulfidophilum* grown in POME (cPOME-PD1) or biomass of *Rhodovulum sulfidophilum* grown in POME (bPOME-PD1) in grey tank for 30 days. Initial stocking size in 25 L = 375 larvae. *Artemia* nauplii only introduced from Day 21.

Day of culture	Live feed fed with cPOME-PD1 (Treatment 4a)	Live feed fed with bPOME-PD1 (Treatment 4b)
	% survival (from Day 0)[1]	
0	100.0 ± 0.0	100.0 ± 0.0
10	95.7 ± 5.0	91.4 ± 11.2
20	84.5 ± 4.1	76.7 ± 23.8
30	81.9 ± 3.0	71.4 ± 20.5
	Mean TL of larvae (mean mm ± SD)[2]	
0	3.73 ± 0.06	3.73 ± 0.06
10	5.56 ± 0.46	5.49 ± 0.25
20	9.14 ± 0.08	8.83 ± 0.42
30	11.07 ± 0.05	12.30 ± 0.52
	Mean daily growth rate (mmTL/day ± SD)[2]	
0-10	0.18 ± 0.05	0.18 ± 0.02
10-20	0.36 ± 0.05	0.33 ± 0.03
20-30	0.19 ± 0.01	0.35 ± 0.09

[1] Mean of triplicate values; [2] Mean of nine replicate values; see Appendix XXVI for detailed statistical test of significance

7.3.5 Water quality

The recorded water quality and environmental parameters among treatments and experiments were not significantly different ($P > 0.05$). In all four experiments, the ammoniacal-nitrogen, nitrite and nitrate concentrations ranged from 0.01 to 0.62 mg/L, 0.009 to 0.924 mg/L and 0.10 to 2.17 mg/L respectively, while the water pH, DO, conductivity and temperature fell within the range of 6.94 to 9.06, 4.20 to 7.49 mg/L, 9.06 to 10.17 mS and 26.6 to 30.4 ℃ respectively (Table 7.5).

Table 7.5 Water conditions of the culture water of marble goby larvae

Day	Parameter[1]	Treatment 1a	Treatment 1b	Treatment 1c	Treatment 2a	Treatment 2b	Treatment 3a	Treatment 3b	Treatment 3c	Treatment 4a	Treatment 4b
0	Ammoniacal-nitrogen (mg/L)	0.32	0.44	0.58	0.21	0.14	0.11	0.07	0.08	0.07	0.06
	Nitrite (mg/L)	0.082	0.114	0.122	0.014	0.014	0.010	0.012	0.009	0.011	0.011
	Nitrate (mg/L)	0.64	0.77	0.84	0.37	0.40	0.31	0.27	0.35	0.52	0.55
	pH	7.56	7.53	7.59	7.32	7.41	7.36	7.41	7.35	7.32	7.64
	DO (mg/L)	5.29	5.34	5.25	5.48	5.48	4.90	4.89	4.68	5.44	5.34
	Conductivity (mS)	9.18	9.61	9.09	9.87	9.82	9.10	9.33	9.19	9.12	9.06
	Temperature (℃)	28.7	28.6	28.6	27.5	27.4	29.6	29.5	30.4	27.3	27.2
10	Ammoniacal-nitrogen (mg/L)	0.45	0.62	0.33	0.54	0.20	0.03	0.25	0.05	0.17	0.02
	Nitrite (mg/L)	0.030	0.130	0.021	0.021	0.009	0.024	0.079	0.021	0.025	0.017
	Nitrate (mg/L)	0.25	0.47	0.20	0.32	0.24	0.14	0.33	0.18	0.24	0.25
	pH	7.47	7.44	7.46	7.24	7.30	9.06	7.46	8.17	8.09	8.67
	DO (mg/L)	5.07	5.01	5.04	5.05	5.13	5.89	5.20	5.00	5.06	5.85
	Conductivity (mS)	9.88	9.83	9.85	9.64	9.61	9.89	9.88	9.96	9.66	9.59
	Temperature (℃)	30.1	29.8	30.0	28.3	28.3	28.7	28.1	29.2	28.7	28.7
20	Ammoniacal-nitrogen (mg/L)	0.16	0.13	0.11	0.39	0.13	0.10	0.41	0.07	0.01	0.07
	Nitrite (mg/L)	0.030	0.124	0.018	0.044	0.012	0.018	0.410	0.016	0.018	0.011
	Nitrate (mg/L)	0.28	0.49	0.23	0.30	0.23	0.10	1.09	0.14	0.20	0.18
	pH	7.63	7.51	7.63	6.98	6.99	8.80	7.50	8.60	8.19	8.34
	DO (mg/L)	5.76	5.58	5.62	5.07	4.96	6.43	5.02	6.14	5.73	6.09
	Conductivity (mS)	9.49	9.49	9.47	9.58	9.57	9.87	9.92	10.08	9.80	9.71
	Temperature (℃)	26.9	26.8	26.8	27.4	27.4	29.1	28.9	30.1	28.4	28.4

Table 7.5, continued

Day	Parameter[1]	Treatment 1a	Treatment 1b	Treatment 1c	Treatment 2a	Treatment 2b	Treatment 3a	Treatment 3b	Treatment 3c	Treatment 4a	Treatment 4b
30	Ammoniacal-nitrogen (mg/L)	0.03	0.02	0.02	0.11	0.03	0.58	0.52	0.10	0.11	0.10
	Nitrite (mg/L)	0.027	0.033	0.013	0.204	0.029	0.026	0.924	0.030	0.027	0.026
	Nitrate (mg/L)	0.22	0.26	0.20	0.66	0.27	0.14	2.17	0.22	0.20	0.25
	pH	8.49	8.23	8.33	7.07	6.94	7.39	7.34	7.01	6.94	7.06
	DO (mg/L)	5.38	5.32	5.35	4.72	7.49	5.57	5.57	4.32	4.20	4.24
	Conductivity (mS)	9.42	9.50	9.45	9.67	9.71	10.17	9.98	9.64	9.81	9.62
	Temperature (℃)	26.8	26.8	26.8	27.5	27.4	26.6	26.8	27.1	27.6	27.7

[1] Mean of triplicate values

7.3.6 Fatty acid and amino acid profiles of freeze-dried rotifers and marble goby larvae

Rotifers fed with cPOME-PD1 recorded higher percentages of protein, lipid and energy than those fed with bPOME-PD1 although both had equal amounts of carbohydrate and ash (Table 7.6). The fatty acid profile reveals that rotifers fed with cPOME-PD1 had significantly higher total PUFA especially n-6 PUFA but lower total SFA and MUFA compared to those fed with bPOME-PD1. Further, rotifers fed with cPOME-PD1 had significantly higher amounts of DHA and ARA than those fed with bPOME-PD1. On the other hand, their percentage of total amino acids was not significantly different ($P > 0.05$).

Fish larvae given rotifers fed with cPOME-PD1 had significantly higher percentages of carbohydrate and ash than those given rotifers fed with bPOME-PD1 but both had about equal amounts of lipid and energy (Table 7.6). However, the former had more protein than the latter. Fatty acid profile demonstrates that fish larvae given rotifers fed with bPOME-PD1 had significantly higher amount of n-3 PUFA especially DHA and EPA than those given rotifers fed with cPOME-PD1 but both enriched larvae had equal total PUFA (Appendix XXIIa). The former also had significantly higher amount of total amino acids than the latter (Table 7.6; Appendix XXIIb).

Table 7.6 Proximate composition (%), fatty acids (% total fatty acids) and amino acids (% protein) of freeze-dried rotifers and marble goby larvae (mean of duplicate values)

Proximate profile	Rotifers fed with bPOME-PD1	Rotifers fed with cPOME-PD1	Fish larvae given rotifers fed with bPOME-PD1	Fish larvae given rotifers fed with cPOME-PD1
Proximate composition (%)				
Protein	47.6	60.0	48.1	34.0
Lipid	6.7	12.8	5.7	5.3
Carbohydrate	5.5	5.3	9.1	20.5
Ash	10.6	10.5	9.0	11.8
Moisture	29.6	11.4	28.1	28.4
Energy, Kcal/100g	272 (1142kJ)	376 (1579kJ)	280 (1176kJ)	266 (1117kJ)
Fatty acids				
ARA	0.66	1.88	0.10	2.30
EPA	2.59	1.57	2.11	1.23
DHA	1.20	2.70	8.35	4.95
Total SFA	41.20	36.96	49.94	43.09
Total MUFA	40.93	29.07	25.47	32.85
Total PUFA	17.87	33.97	24.59	24.06
Percentage total fatty acids	100.00	100.00	100.00	100.00
n-3 PUFA	5.44	4.60	14.51	6.54
n-6 PUFA	12.02	24.62	9.32	17.20
ARA/EPA	0.26	1.20	0.05	1.88
DHA/EPA	0.46	1.72	3.95	4.04
Amino acids				
Total essential amino acids	20.89	26.26	23.72	14.83
Total non-essential amino acids	23.58	27.99	23.63	16.47
Percentage total amino acids	44.47	54.25	47.35	31.29

See Appendix XVIII and XXII for detailed statistical test of significance

Biplots of the samples and the types of fatty acids and amino acids they contained were derived from PCA (Figure 7.2). The first and second PCA axis explained 50.7% and 31.7% of the total variation, respectively. The rotifers and larval fish tested contained significant amount of LNA, LA, EPA, DHA and ARA. Fish larvae had higher DHA but lower EPA compared to their diets (Figure 7.2). For amino acids,

fish larvae contained lesser amounts of valine, phenylalanie, arginine, serine, proline and tyrosine than their diet. However, fish larvae had higher amount of methionine than their diets (Figure 7.2).

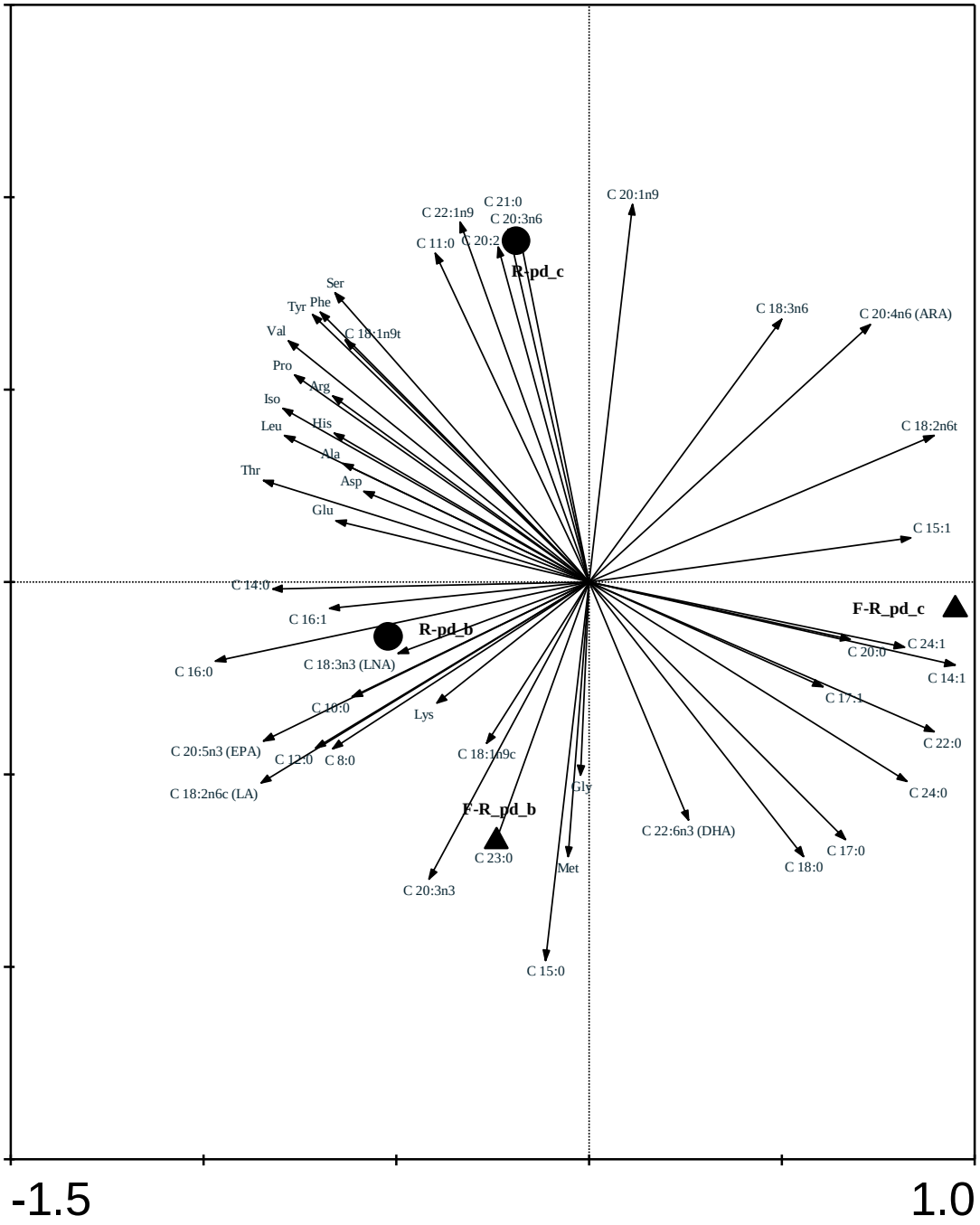


Figure 7.2 PCA of the fatty acid and amino acid compositions of freeze-dried rotifers and marble goby larvae. Samples (filled circles, triangles): R-pd_b = Rotifers fed with bPOME-PD1, R-pd_c = Rotifers fed with cPOME-PD1, F-R_pd_b = Fish larvae given rotifers fed with bPOME-PD1, F-R_pd_c = Fish larvae given rotifers fed with cPOME-PD1; Variables (Arrows): 37 fatty acids and 16 amino acids in conventional notations

7.4 Discussion

The present study has demonstrated the significantly improved survival and growth of marble goby larvae when they were given a diet of live feed (rotifers and *Artemia* nauplii) fed with bPOME-PD1 or cPOME-PD1. Larval rearing was more successful in grey coloured tank with a stocking density of 15 larvae/L. High stocking density resulted in high mortality and poor growth of fish larvae (see Table 7.1). This is likely due to the greater stress borne by larvae at higher density attributed to deterioration of water quality, limited space as well as adverse social interactions including cannibalism, aggressiveness and the hierarchical phenomenon (Pickering & Pottinger, 1989; Procarione et al., 1999). In the present study, the water quality is unlikely the cause of high mortality in high density (see Table 7.5) as the ranges fell within the recommended safe levels (Cheah et al., 1994), with regular water exchanges. Food supply is also not an issue as the prey density was regularly maintained at 10 prey/mL. Rather, the food provided was more than the larval fish required. A marble goby larva of age 18 dph consumed 147.4 rotifers/day, while a larva of age 27 dph consumed 43.4 rotifers/day and 197.6 *Artemia*/day (Amornasakun et al., 2003). The maintained feed density and water quality seemed to support good larval survival at a stocking density of 15 and 20 larvae/L. However, since the growth rate of larvae at 20/L was significantly lower ($P < 0.01$) than at 15/L (see Table 7.1), the latter is taken as the optimal stocking density for rearing marble goby larvae. This stocking density was thus subsequently used in latter experiments (see Table 7.2 – 7.4).

Although larval fish given live feed fed with cPOME-PD1 had significantly higher survival than those given live feed fed with POME (see Table 7.2), the study shows that POME is a nutrient-rich waste. POME is rich in lipids, protein, carbohydrate, minerals and nitrogenous compounds (Phang, 1990). The use of PB with POME as rotifer feed improves the nutritional profile of rotifers (see Table 7.6), as

further attested by higher survival of larvae given live feed fed with cPOME-PD1 compared to those given live feed fed with POME. In fact, larval survival was even higher when given a diet of live feed fed with cPOME-PD1 (60.9%; see Table 7.2) as compared to those given live feed fed with bPOME-PD1 (42.0%; see Table 7.1), although the latter gave higher growth rate than the former. However, a further confirmatory test demonstrates that live feed fed with bPOME-PD1 or cPOME-PD1 was equally good in sustaining larval survival and growth (see Table 7.4). Larval survival is even better than those given live feed fed with *Nannochloropsis* sp. and cod oil juice at 5 ppt salinity (Senoo et al., 2008).

The good survival and growth of larvae are likely due to the suitable DHA/EPA and ARA/EPA ratios in the larvae, attributable to the given diet. Previous study (Chapter 6) had shown that larvae with low ratios of DHA/EPA (3 – 4) and ARA/EPA (0.1 – 3) survived better than those with high ratios of DHA/EPA (> 11) and ARA/EPA (> 5) (see Table 6.5, page 117). In the present study, good survival and growth are also recorded in larvae with low ratios of DHA/EPA (3 – 4) and ARA/EPA (0.1 – 2) (see Table 7.6). Hence, it is suggested that a DHA:EPA:ARA ratio of approximately 7:2:1 is likely the optimal HUFA ratio for marble goby larvae.

In addition, C16:0, C18:0 and C18:1n9c were also found to be predominant in larvae given live feed fed with cPOME-PD1 and bPOME-PD1. These SFA and MUFA are usually the preferred elements for mitochondrial β-oxidation (Andrade et al., 2003) and also used to generate metabolic energy in fish via the TCA cycle (Henderson & Sargent, 1985). The high ratio of DHA/LNA in fish larvae (13.75 – 23.86) as compared to their diet (1.88 – 7.94) clearly demonstrates the larva's bioconversion ability (see Table 7.6). This confirms that marble goby larva is able to bioconvert LNA in its diet to DHA.

The right colour of larval-rearing tanks could have direct influenced on larval foraging, survival and growth (Puvanendran & Brown, 2002; Monk et al., 2006), although the response of larval fish to tank colour is highly variable and not fully understood due to the confounding effects of different feeds, photoperiod and stocking density (Bradner & McRobert, 2001). In the present study, fish larvae cultured in grey tank had the highest survival throughout the rearing period when compared to black and transparent tanks (see Table 7.3). The high survival of larvae reared in grey tank was also observed in Experiment 4 regardless of the type of feed (see Table 7.4). Thus, the present findings do not totally accept the hypothesis that the dark coloured tank supports higher survival of larval marble goby as compared to the light coloured tank. High survival of fish larvae reared in grey tanks is likely the result of a suitable tank background (given its grey colour) that enables better prey detection and hence, greater food intake by larvae. For instance, high visual contrast of prey provided by a dark background enhanced prey capture and consumption in yellow perch *Perca flavescens* (Hinshaw, 1985) and striped bass, *Morone saxatilis* (Martin-Robichaud & Peterson, 1998). It is not obvious why grey is better than black tank since the latter had been reported to provide a good contrast between food and background, as well as an illusion of a natural environment to fish (Nass et al., 1996). However, it is obvious that the transparent tank has an excessively bright environment that not only stresses fish larvae but also interferes with larval vision leading to low prey capture (Höglund et al., 2002; Rotllant et al., 2003). Nonetheless, the larvae of marble goby, being positively phototactive (Senoo et al., 1994c), tend to aggregate at the water surface where they are bubbled out of the water, become stuck to the sides of the tank and then died. Thus, in dark background tank, fish will distribute more homogeneously throughout the water column (Martin-Robichaud & Peterson, 1998).

In conclusion, the significantly improved survival and growth rate of marble goby larvae given rotifers and *Artemia* nauplii fed with POME-PD1 and reared in grey coloured tank, with the fish stocking density of 15 larvae/L and in 5 ppt salinity had warranted a cheap and stable mass culture system for marble goby larvae. The use of rotifers and *Artemia* nauplii fed with cPOME-PD1 or bPOME-PD1 to give a ratio of DHA:EPA:ARA of ca. 7:2:1 as fish feed is recommended as it gave good survival and growth to marble goby larvae.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

This final chapter covers three main topics of discussion as a result of this study. These are a) a proposal of a large scale mass production of POME-PD1; b) a basic guidance for farmers in rearing larval marble goby; and c) a brief cost analysis of mass production of POME-PD1 and larval marble goby.

8.1 Pilot scale mass production of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) for aquaculture

Overall, the present study has shown that POME-PD1 is a potential aquaculture feed for the future. Based on the success of the present study, production of POME-PD1 on a pilot commercial scale is the next step. The POME-PD1 can easily be produced in ziplock bags and the PD1 cultured in POME is projected to give a dry cell biomass of 2.06 g/L after 60 h of inoculation (see Section 4.3.5, page 73). Therefore, 1 g of bacterial dry biomass could be extracted from 121.36 mL of POME.

An evaluation of the different methods of product development for POME-PD1 and determination of the best method in terms of product form, delivery to organisms, preservation, shelf life and packaging, however, is first needed. It is also necessary to further test the versatility of POME-PD1 as feed for other zooplankton species (e.g. copepods and *Moina* sp.), as well as direct or indirect feed for other commercial species of fish (e.g. tiger and giant groupers) and prawns. Such testings are necessary to assess the universal use of POME-PD1 and increase its marketability as a feed for zooplankton and larval fish.

The strategies to be taken to make POME-PD1 more competitive as the future zooplankton and fish feed, are: a) universal use and its value for money: hence, further feeding trials of POME-PD1 on rotifers, zooplankton and commercial fish and prawn

species, on a pilot commercial scale; b) convenience and availability on demand: further product development of POME-PD1 as an aquaculture feed, including product form, shelf life, etc. is necessary; and c) economical: the commercial production of POME-PD1 to be made cost effective and cheap to aquaculturists. Figure 8.1 illustrates a flow diagram of how further research should be designed to commercially produce POME-PD1 based on the above strategies.

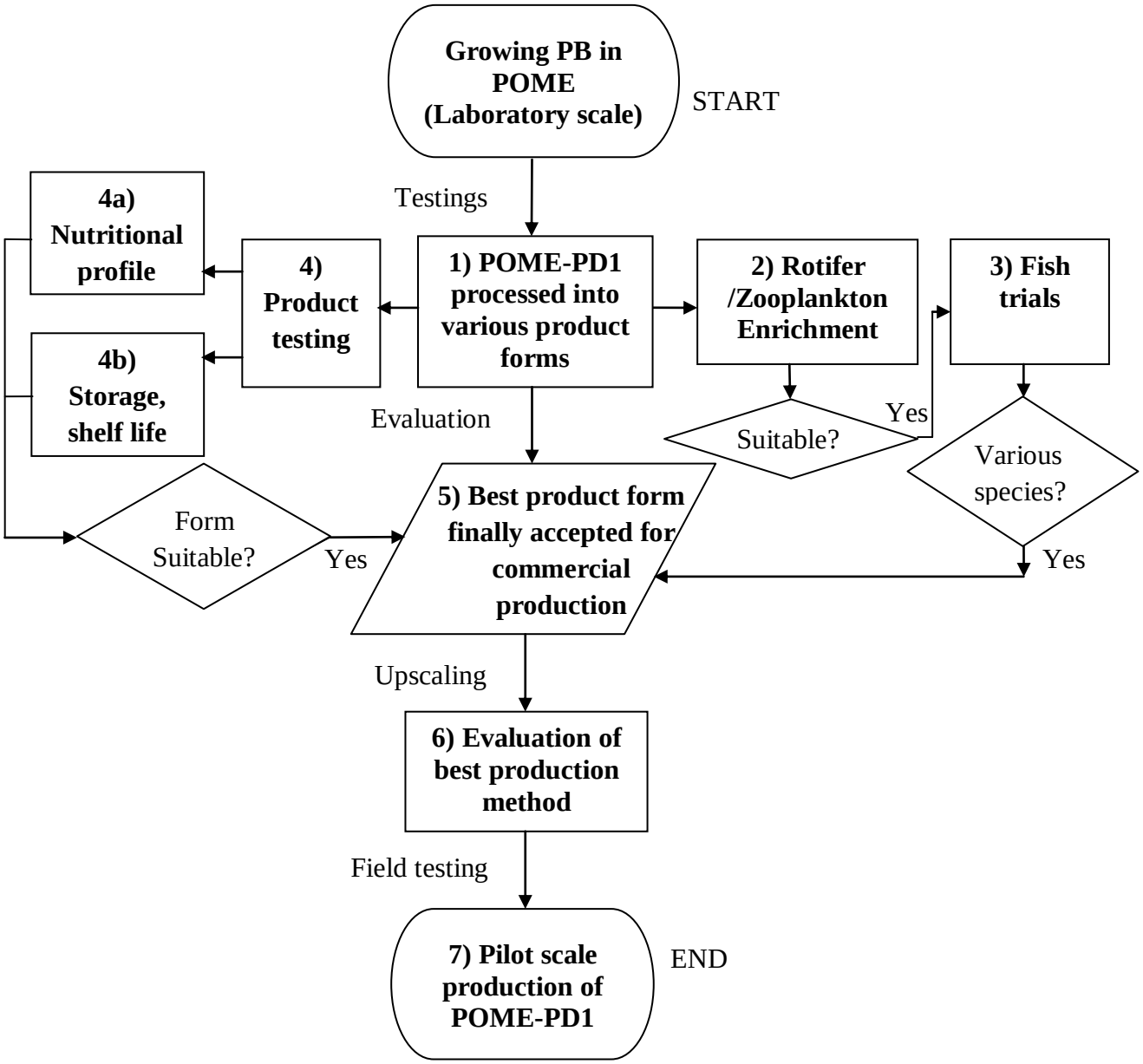


Figure 8.1 A schematic flow chart of further research and the mass production of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) for aquaculture

Explanation to steps:

START – Production of POME-PD1

1) Production of POME-PD1 on laboratory scale for testing of several product forms which include: a) Freeze dried; b) Frozen; c) Oven dried pellets; d) Nano spray dried; e) Drum dried; and g) Pulse combustion dried

2) Testing several POME-PD1 product forms on the production and fecundity of rotifer and other zooplankton

3) Testing rotifers fed with POME-PD1 (different forms) as feed for commercially important larval prawn and fish such as tiger and giant groupers

4a) Evaluating the nutritional profile of the various POME-PD1 product forms:

a) Carbohydrate; b) Protein; c) Lipid; d) Fatty acids; and e) Amino acids

4b) Determine the best method to preserve and store POME-PD1 using various types of protective agents, sugars and skim milk powder

5) Selection of the best POME-PD1 product form in terms of survival and growth of fish and prawn, storage and convenient for delivery method mass production

6) Evaluation of POME-PD1 production in large plastic pillows against bioreactors, in terms of biomass production and costs

7) POME-PD1 consistently produced on a pilot commercial scale. END OF PROCESS.

8.2 Hatchery production of larval marble goby: A guide for farmers

8.2.1 Selection and maintenance of marble goby broodstock

Both wild caught and domesticated or reared marble goby adults can be used as broodstock but normally wild fish are preferable to hatchery-reared fish. However, the use of hatchery-reared fish enables the selection of good quality fish with respect to fast growth, resistance to disease, etc. (Alessandro et al., 1999). Sufficient quantity and high

quality eggs can be obtained from a female marble goby of about 100 to 150 g in body weight (Senoo et al., 1993b).

The broodstock can be fed with live koi and cockle at a feeding rate of 3% body weight of broodstock, once a day. This is because koi is rich in protein (ca. 50%) (Farhat & Abdul Shakoor, 2011), while cockle is rich in PUFA (ca. 50%) (Copeman & Parrish, 2004). Koi should be salt bathed in 5 ppt salinity for 10 min prior to feeding the broodstock. A diet containing reasonable amounts of PUFA, protein and vitamins for feeding of broodstock is vital in obtaining high quality eggs and healthy larvae.

Marble goby can spawn all year round under optimum environmental conditions (Tavarutmaneegul & Lin, 1988). It was reported that marble goby fish spawned naturally in good weather and at water temperatures that ranged from 27 to 32 ℃ (Senoo et al., 1994a). Marble goby also breeds intensively during the rainy season (Sompong, 1980). We have recorded a total of 82 times of natural spawning from seven broodstock pairs from 1st October 2010 to 30th September 2011. The highest spawning frequency was recorded in August 2011, whereas the lowest spawning frequency was in February 2011 (Figure 8.2). Low spawning can be overcome by inducing spawning using spawning hormones such as HCG and carp pituitary glands (PG). Only a single injection is required to artificially induce spawning of broodstock where 1 kg female will receive 1000 IU of HCG, while the male will receive half of that dosage. This method may be a necessity for mass culture of marble goby larvae on a large scale.

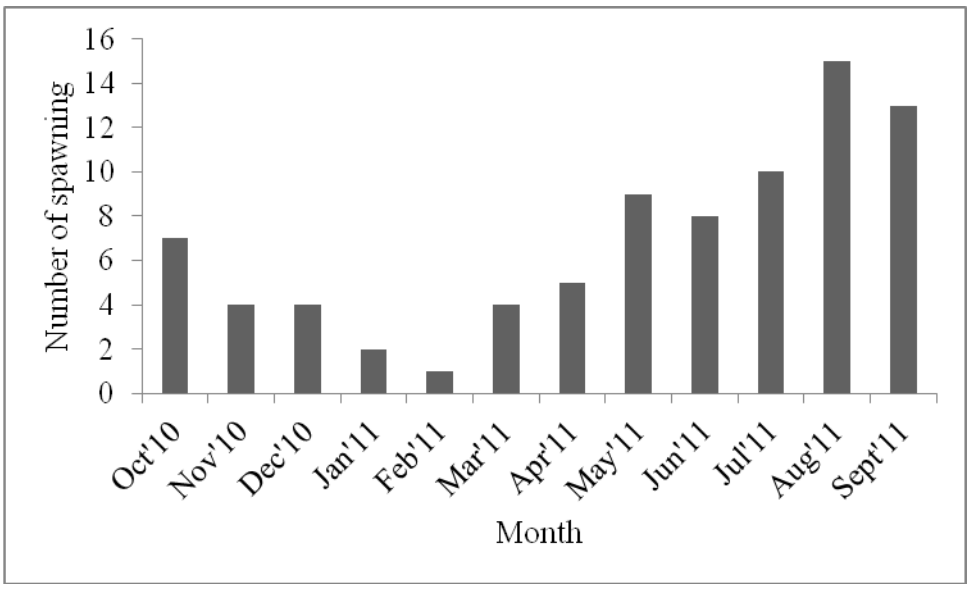


Figure 8.2 The spawning frequency of marble goby over a year (number of pair = 7)

Diseases can slow down the process of large scale mass culture of larval marble goby. Anchor worms (*Lernaea cyprinacea*), a parasitic cyclopoid copepod and epizootic ulcerative syndrome (EUS) or 'red spot' commonly infected the marble goby adults in the culture system. Although there are a number of ways to eliminate the anchor worms such as using formalin and Dipterex (Mahmoud & Layla, 2003), the most effective treatment we have tested was using 10 ppt salinity. Salt bath treatment is recommended as it is safer, environmental friendly and economical compared to chemicals. The infected fish should be kept in 10 ppt salinity until recovery. The salinity of culture water should immediately be brought back to freshwater after the broodstock has recovered.

Epizootic ulcerative syndrome caused by a pathogenic oomycete fungus, *Aphanomyces invadans* is also commonly encountered in marble goby cultures. The infected fish had ulcerative lesions on their body and/or head. This disease has been reported to commonly occur during the rainy season and cooler weather (Prasankok et al., 2002). Malachite green was reported to be effective in treating the infected fish

(Campbell et al., 2001). However, we found that salt solution (5 - 10 ppt salinity) effectively treated the infected fish.

8.2.2 Egg management

The fertilized eggs collected from a broodstock tank should be transferred to a new culture medium and the temperature of the new culture water should be adjusted similar to the temperature of broodstock tank in order to prevent the fertilized eggs from temperature shock. The male parent should also be transferred as it guards and fans the fertilized eggs until hatching. Fanning of the fertilized eggs promotes better egg development. When all the eggs are fully hatched out after 1 to 2 days of incubation, the male is transferred back to its tank, while the larvae culture water should be increased to 5 ppt salinity. Live feed should be immediately introduced into the culture.

8.2.3 Rearing of larval marble goby

Marble goby larvae should be fed with live food of suitable sizes according to their age (Table 8.1) or mouth gape. These feeds should be fed at least two days prior to larval feeding.

Table 8.1 Sequence of live foods provided during cultivation of larval marble goby

Day post-hatch	Food	Feeding density
1 – 10	*Brachionus rotundiformis*	10 ind/mL
11 – 20	*Brachionus rotundiformis*	10 ind/mL
21 – 30	*Brachionus rotundiformis*	5 ind/mL
	2-day old *Artemia* nauplii	5 ind/mL
31 – 40	2-day old *Artemia* nauplii	10 ind/mL
40 – 50	2-day old *Artemia* nauplii	5 ind/mL
	Moina sp.	5 ind/mL
50 - 60	*Moina* sp.	10 ind/mL

Rotifer (*Brachionus rotundiformis*) is the primary and favoured feed for culturing marble goby. However, rotifer cultures often 'crash' or show rapid decrease in production which in turn can affect the mass culture of larval marble goby. Hence, there is the need to establish a cheap yet stable mass culture technique for producing highly nutritious rotifers in the shortest time.

We noticed that a minimum initial rotifer stocking density of 75 ind/mL should be used for mass culturing rotifers since low initial rotifer density did not increase rotifer production (Table 8.2). The rotifer culture used as an inoculum should be at the mid-exponential stage with a minimum of 20% fertility rate, calculated based on the percentage of total egg sacs over the total rotifers (Alessandro et al., 1999). In our hatchery, the pure strain of rotifer was cultured in small beakers and maintained at 1 or 2 ind/mL in 5 ppt salinity prior to its use as a starter for new cultures. They were given a low density feed of bPOME-PD1.

Table 8.2 Effect of initial stocking density on rotifer reproduction

Day of culture	25 ind/mL	50 ind/mL	75 ind/mL	100 ind/mL
0	25 ±0	50 ±0	75 ±0	100 ±0
1	27 ±5	53 ±9	88 ±8	118 ±13
2	34 ±8	64 ±8	102 ±6	124 ±5
3	22 ±5	46 ±9	60 ±7	78 ±22
4	1 ±1	1 ±1	5 ±6	5 ±6

Further, the rotifer is a very versatile species as it can survive in a wide range of salinities, ammoniacal-nitrogen concentrations and temperature. Mass culture of rotifers fed with POME-PD1 has been successfully produced in 5 ppt salinity (see Figure 5.2 – Figure 5.4). Rotifers should be cultured within the salinity of range 5 to 10 ppt but not at higher salinities (Table 8.3). The optimum reproductive rate is achieved under restricted environmental conditions and is correlated with the selected rotifer strain and feed type. We noticed that rotifers given a diet of cPOME-PD1 reproduced successfully under the

conditions as shown below (Table 8.4). Hence, these conditions are recommended as optimum for culturing rotifers fed with cPOME-PD1.

Table 8.3 The production of rotifer (ind/mL) in four different salinities

Day of culture	5 ppt salinity	10 ppt salinity	15 ppt salinity	20 ppt salinity
0	1 ±0	1 ±0	1 ±0	1 ±0
1	1 ±0	2 ±1	2 ±1	2 ±1
2	2 ±2	2 ±1	2 ±1	2 ±1
3	7 ±2	7 ±1	3 ±1	3 ±2
4	11 ±6	12 ±3	5 ±2	6 ±2
5	15 ±5	17 ±6	5 ±2	9 ±1
6	3 ±1	12 ±3	6 ±2	3 ±2

Table 8.4 Optimum rearing conditions for rotifers given a diet of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) in a batch culture system

Parameter	Recommended condition[1]
Feed (cPOME-PD1)	ca. 67 mL or 0.112 g DW of bacterial food per 1 L of culture water
Rotifer stocking density	Minimum 75 ind/mL
Salinity (ppt)	5 - 10 ppt salinity
pH	6.5 - 7.0
DO (mg/L)	Minimum 1
Conductivity (mS)	11 - 13
Temperature (℃)	27.0 - 30.0
Ammoniacal-nitrogen (mg/L)	< 7.00
Nitrite (mg/L)	< 0.007
Nitrate (mg/L)	< 0.2

[1] Adapted from Table 5.5 - Treatment 3a (Day 0 - Day 4) (page 96)

In recent trials, we noticed that the marble goby larvae preferred live feed (e.g. rotifers and *Artemia* nauplii) over pellet feed. The use of nano-pellet (Omega rich: a mixture of fish meal, fish oil, soy protein, essential minerals and vitamins; protein: 42%, lipids: 8%) as a feed for newly-hatched marble goby larvae had resulted in total mortality, although the size of the pellet is about the size of the rotifer. Fish larvae only survived up to 14 days of cultivation, whereas those given live feed (rotifers and *Artemia* nauplii) fed with cPOME-PD1 survived until the end of the rearing period of 30 days (about 55% survival). The larvae were cultured in grey tanks and 5 ppt salinity at a

stocking density of 20 larvae/L. We also found out that feeding of instant microalgae to larvae resulted in total mortality after 10 days of cultivation (see Table 6.1, page 110).

Culture conditions that are unfavourable to larval marble goby can lead to massive mortality. We have successfully identified the optimal environmental conditions for rearing larval marble goby based on our present findings (Table 8.5).

Table 8.5 Environmental parameters for rearing larval marble goby

Parameter	Recommended condition[1]
Feed	Live feed fed with cPOME-PD1 or bPOME-PD1
Fish stocking density	15 larvae/L
Culture tank	Grey colour tank
Salinity (ppt)	5 ppt salinity
pH	6.90 – 8.70
DO (mg/L)	4.20 – 6.10
Conductivity (mS)	9.10 – 9.90
Temperature (℃)	27.0 – 29.0
Ammoniacal-nitrogen (mg/L)	< 0.20
Nitrite (mg/L)	< 0.030
Nitrate (mg/L)	< 0.60

[1] Adapted from Table 7.5 – Treatment 4a and Treatment 4b (Day 0 – Day 30) (pages 140 – 141)

8.3 Simple benefit-cost analysis of production of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) and marble goby

8.3.1 POME-PD1

One kilogram DW of bacteria can be produced based on a total cost of MYR48.54 (1 USD = 3.15 MYR) and one only needs POME, water and simple culture equipment (Table 8.6) as compared to other similar feed products available in the market (Table 8.7). We found out that 1.344 g of POME-PD1 dry biomass could produce a culture of ca. 900 rotifers/mL after 4 days of cultivation in a working volume of 3 L (see Figure 5.4, page 88). Therefore, the amount of POME-PD1 required to produce one metric ton of rotifers at this density is 448 g bacterial dry biomass. The cost to support this production is MYR21.75 (0.448 x MYR48.54) for POME-PD1, whereas

Culture Selco Plus costs MYR157.15 (0.449 x MYR350) and Instant Microalgae (*Nannochloropsis* sp.) costs MYR817.25 (2.335 x MYR350). Thus, POME-PD1 is value-for-money since it can produce rotifers cheaper than other feeds (Table 8.7).

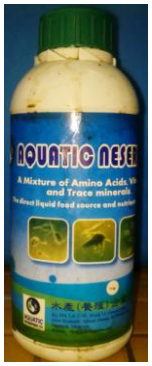
One metric ton of rotifers could daily support 1,347,000 marble goby larvae. Hence, the challenge is to produce the POME-PD1 on a large scale so that it could be harvested and packed commercially for immediate use in the hatchery for feeding rotifers.

Table 8.6 An estimated cost to produce 1 kg of freeze dried *Rhodovulum sulfidophilum* grown in POME (POME-PD1)

Phototrophic bacterium	Chemicals/Materials	Cost (MYR)[a]
a) Inoculum (112-PD1)	Dipotassium phosphate (K_2HPO_4)	0.025
	Magnesium sulphate ($MgSO_4.7H_2O$)	0.020
	Yeast extract	0.050
	NaCl	0.025
	1 L water	0.015
	Electricity etc	0.035
	Total	0.170
b) To produce POME-PD1	10% of 112-PD1 (inoculum)	0.017
	75% water	0.011
	Electricity, ziplock bag, 25% POME etc	0.050
	Freeze drying POME-PD1	0.022
Total cost to produce 2.06 g/L of bacterial dry cells after 60 h of cultivation		0.100
c) Cost of 1 kg of bacterial dry cell biomass = (0.10 x 1000)/2.06		**48.54**

[a] 1 USD = 3.15 MYR

Table 8.7 The price of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) as compared to some commercial zooplankton feed products

	Local		International		
Product					
	POME-PD1	Aquatic Nursery Dew	*Nannochloropsis* sp.	Culture Selco Plus	Easy Selco
Price	MYR48.54/kg DW	MYR 200/600 mL	MYR 350/L (ca. 18.4% of DW)	MYR350/kg DW	MYR 300/1.2 L

8.3.2 Marble goby

Marble goby is preferably sold live as the price of chilled dead fish costs 30 to 50% less than the live fish (Jatuporn, 2007). The demand for this species is high regardless of the domestic or international market. In Malaysia, the price of 1 kg live marble goby is MYR70 to MYR80, while one inch (2.54 cm) marble goby fingerling is sold at MYR1.50/piece. The main importers of live marble goby are Taiwan (preferable 400 – 800 g), Singapore and Hong Kong (800 g and above) (Jatuporn, 2007).

A simple benefit-cost projection for the mass production of one inch marble goby fingerlings shows that the marble goby industry can be profitable (Table 8.8). This projection is based on a small hatchery of 36 m x 15 m size, capable of producing 0.92 million fingerlings in a year. The first month is for setting up all the facilities for larval nursing. The initial capital investment is estimated at MYR113,000 which includes larval and rotifer rearing tanks, large plastic pillows to culture POME-PD1, generator set, air blowers, power installation, submersible pumps and others (marble goby broodstock, reservoir tanks, accessories and induced spawning hormone such as HCG).

Table 8.8 A simple benefit-cost projection based on hatchery size of 36 m x 15 m

Month	Production of marble goby fingerlings				Cost production		Net profit
	Spawns (one batch = eight thousand of one inch fingerlings)	Estimated number of fingerlings produced	Sales (MYR1.50/fingerling)	Total (cumulative) (MYR)	Total (MYR)	Total (cumulative) (MYR)	
First month	0	0	0	0	113,000	113,000	-113,000
Second month	10	0	0	0	13,000	126,000	-126,000
Third month	10	0	0	0	13,000	139,000	-139,000
Fourth month	10	0	0	0	13,000	152,000	-152,000
Fifth month	15	80,000	120,000	120,000	16,000	168,000	-48,000
Sixth month	15	80,000	120,000	240,000	16,000	184,000	56,000
Seventh month	15	80,000	120,000	360,000	16,000	200,000	160,000
Eighth month	20	120,000	180,000	540,000	19,000	219,000	321,000
Ninth month	20	120,000	180,000	720,000	19,000	238,000	482,000
Tenth month	20	120,000	180,000	900,000	19,000	257,000	643,000
Eleventh month	25	160,000	240,000	1,140,000	22,000	279,000	861,000
Twelfth month	25	160,000	240,000	1,380,000	22,000	301,000	1,079,000

The production of larval marble goby begins at the second month with a minimum target of ten spawning and the number of spawning increases when the operation becomes more stable. The initial production cost is estimated at MYR13,000 which includes cost to produce POME-PD1, seawater transport (MYR650 per trip), feed for marble goby broodstock, staff, utilities, etc. In the subsequent months, the production cost increases as the intended number of spawning is increased. A maximum of twenty five spawning per month is what the hatchery could support for subsequent larval culture. Fish sales only begin at the fifth month as the marble goby is estimated to reach one inch size in ca. three months (95 days) of rearing based on the growth curve (Figure 8.3). Each batch of spawning is estimated to contain ca. twenty thousand fertilized eggs and the hatching rate of 85%.

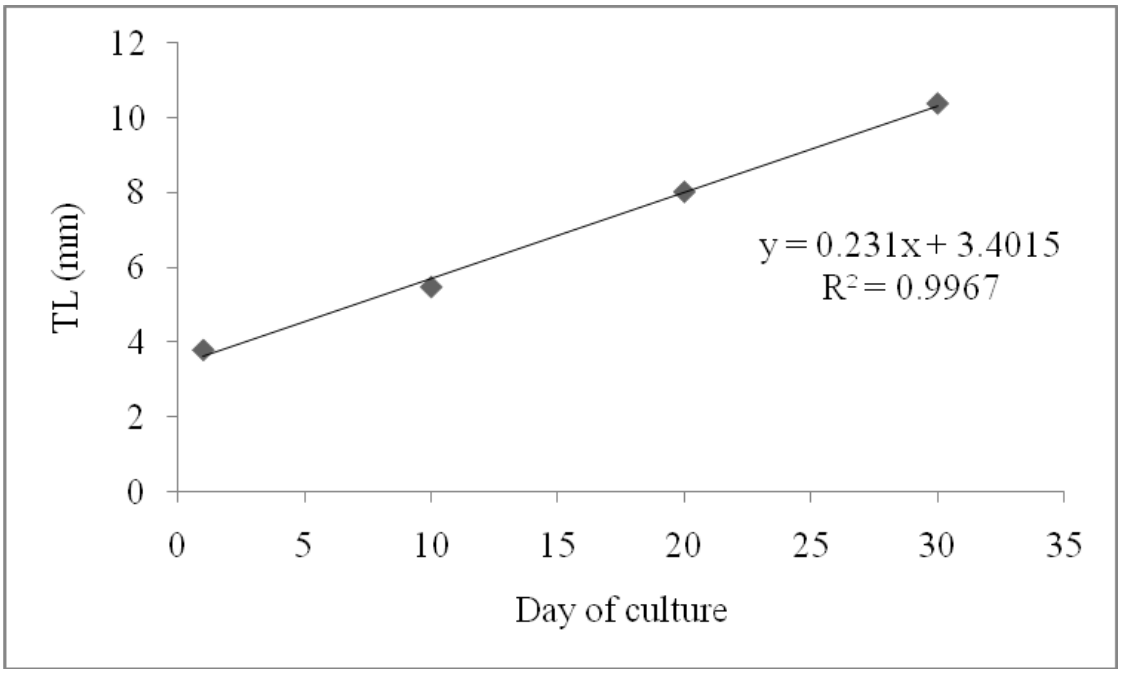


Figure 8.3 Growth (total length - TL, mm) of marble goby larvae given live feed fed with *Rhodovulum sulfidophilum* grown in POME (POME-PD1) in the first 30 days post-hatch (dph)

A minimum of 35% larval survival is expected when the marble goby reaches one inch size in about three months of rearing. This survival is calculated based on an average of 67% survival (n = 5) of 30 dph marble goby larvae (see Table 7.1 - 7.4) and on the assumption of an average of 20.5% and 11.5% mortality, respectively for the second and third month of rearing (Table 8.9).

Table 8.9 The percentage survival of one inch marble goby fingerlings after three months of rearing (this study)

Per batch	% Survival	Number of surviving marble goby
Fertilized eggs	20,000	
Hatching rate	85%	17,000
First month of rearing	67%	11,390
*Second month of rearing	79.5%	9,055
**Third month of rearing	88.5%	8,014

* Tavarutmaneegul & Lin (1998): Survival of 30 to 60 dph larvae = 60 – 99% (Average: 79.5%)
** Muhammad Darwis et al. (2008); Muhammad Darwis et al. (2009): Survival of 40 to 100 dph larvae = 85.6 – 91.3% (Average: 88.5%)

In this analysis, six months are required to recover the expenses (Table 8.8). However, this projection excludes the costs of building the hatchery, including land cost which depends on the locality.

8.4 Limitations of present study

a) The bacterial biomass in PB grown in POME used in the present study is not pure bacteria but a mixture of bacteria and very fine debris of palm material. Only biomass of PB grown in 112 medium is pure.

Although centrifugation of POME and filtration of its supernatant is carried out prior to bacterial inoculation, the filtered supernatant still contains the finest solid debris. One litre of 25% diluted filtered POME (v/v) medium was estimated to contain an average of 0.438 g of dry solid debris (n = 5), and 2.06 g/L of bacterial dry cell biomass was harvested after 60 h of culture in the POME (see Section 4.3.5, page 73).

Thus, the POME-grown bacterial biomass contains ca. 79% bacteria and 21% solid debris.

Although POME alone has been shown to support rotifer production (see Figure 5.4, page 88) and sustain the survival of marble goby larvae via live feed (rotifers and *Artemia* nauplii) cultured in POME (see Table 7.2, page 135), the effect of bacteria has been shown to be more significant than POME. Besides, cultures of rotifers fed with biomass of PB grown in 112 medium (100% pure bacteria) are more dense than those fed with biomass of PB grown in POME (see Table 3.2, page 52). Thus, the PB in POME-grown bacterial biomass is the key player or active constituent enhancing rotifer reproduction and larval survival and growth.

b) The culture of marble goby larvae in this study was limited to the first 30 days after spawning.

The study was limited to this period of culture because the first month is critical for the newly hatched larvae whereby 90% mortality has been reported during the first week of rearing and mortality in the first month was significantly higher than later (Tavarutmaneegul & Lin, 1988). The survival of 30 dph larvae was reported to be ca. 10 to 20% (Tavarutmaneegul & Lin, 1988; Senoo et al., 2008). Success in initiating the first feeding of larvae after complete yolk absorption would give higher subsequent survival. Delayed initial feeding or effectively, unsuitable food or malnutrition, could lead to high larval mortality, attributable to the weak larvae being unable to swim and hunt for food (Dou et al., 2002). Hence, this study had taken the challenge to rear and increase larval survival during the first 30 days.

The survival of cultured larvae post-30 dph is thus unknown. However, previous studies have shown that the later larval stages have good survival; e.g. 30 to 60 dph larvae had 60 to 99% survival (Tavarutmaneegul & Lin, 1988) and 40 to 100 dph larvae

had 85.6 to 91.3% survival (Muhammad Darwis et al., 2008; Muhammad Darwis et al., 2009).

c) The rearing of larval marble goby was tested at two salinities of culture water (5 ppt and 10 ppt salinity).

Higher or lower salinities of culture water as compared to the two salinities tested in the present study could also influence the survival and growth of marble goby larvae. A previous study, which tested on six different salinities, namely 0, 5, 10, 15, 20 and 30 ppt, had shown that 10 ppt salinity was the optimum salinity for culturing larval marble goby, while 0 ppt salinity gave the highest mortality rate (Senoo et al., 2008). The use of a lower salinity in the present study, besides being reported as suitable for rearing larval marble goby, is also due to the use of brackishwater rotifers as larval feed. The rotifers do better in salinity of 5 to 10 ppt (see Table 8.3).

d) The investigation of the effect of tank colour on the survival and growth of larval marble goby was limited to three tank colours.

Although there are many other tank colours that can be used to culture larval marble goby, these tank colours might not show clearly the significant impact of background tank colour (as perceived by the larvae) which depends on light illumination. Black, grey and transparent tanks were chosen because the transparent tank is commonly used for rearing larval marble goby (Senoo et al., 2008), while the black coloured tank is used to compare and contrast the conditions provided in the transparent tank. The grey coloured tank would provide an intermediate effect of background colour somewhere between transparent and black coloured tanks. Thus, these three tank colours are sufficient to provide the contrasting background colour under light/dark condition to test larval survival and growth. The larval rearing

experiments were initially conducted using black coloured tanks since previous similar studies had poor survival and growth of larval marble goby in transparent tanks (Senoo et al., 2008). However, the survival results of our earlier experiments were lower as compared to the later experiments using grey coloured tanks which were subsequently adopted.

8.5 Recommendations for future research

a) To study the possible probiotic properties of *Rhodovulum sulfidophilum* grown in POME (POME-PD1) that could improve the immune system of larval fish and shrimps.

Phototrophic bacteria are known to contain some biological cofactors and active substances that act as probionts (Kobayashi & Kobayashi, 1995). The use of probiotics such as PB could benefit the environment of the culture rearing system, serve as a cheap and non-harmful antibiotic and indirectly improve the health of newly-hatched larval fish (Jose et al., 2006) by enhancing their intestinal microbial balance (Gatesoupe, 1999). For instance, *Penaeus paulensis* larvae survived better when given bacteria as compared to those reared in filtered (< 0.1 μm) seawater (Fabiano et al., 1999).

b) Study on the use of *Rhodovulum sulfidophilum* (PD1) as a bioremediation agent to treat POME and improve water quality of aquaculture system.

Phototrophic bacteria can be used to improve water quality of zooplankton and fish cultures, and indirectly improve larval fish survival and growth. Although the effectiveness of POME-PD1 in reducing the concentrations of ammoniacal-nitrogen, nitrite and nitrate have not been seriously tested during culture of marble goby, the water quality of larvae fed with bPOME-PD1 had low concentrations of ammoniacal-nitrogen, nitrite and nitrate throughout the rearing period (see Table 6.4 – Treatment 1a; 1c, pages 114 - 115).

c) Study on the use of artificial or processed formulated feed containing *Rhodovulum sulfidophilum* grown in POME (POME-PD1) for mass culture of zooplankton (e.g. rotifer, copepods, *Moina* sp.) and larval fish and shrimps

The use of artificial formulated feed is preferable on a large scale production of zooplankton and larval fish and shrimps. The advantages include a) long lasting in terms of storage period and quality of feed; b) cost effective as it requires no extra enrichment for zooplankton cultures; c) avoid deterioration of water quality of rearing cultures; and d) easy transportation and handling. In the present study, rotifers were shown to be able to consume heat-killed bacteria (see Figure 5.2, page 84).

d) Study on the use of zooplankton fed with *Rhodovulum sulfidophilum* grown in POME (POME-PD1) as feed for larval fish of other problematic commercial species such as groupers (tiger and giant) to evaluate the universal use of POME-PD1.

High mortality of larval grouper was reported during their early life stages and this is likely due to unsuitable size of the diet (Duray et al., 1997) and/or 'shock syndrome' attributable to feeding larvae with a HUFA-deficient diet (Cowey & Sargent, 1972). It was reported that the amount of EPA (8.8 - 8.9%) and DHA (0.1 - 5.5%) enhanced the growth and survival of larval tiger grouper (Waspada et al., 1991). The use of zooplankton fed with POME-PD1 could fulfill the lipid requirement of grouper since POME-PD1 is rich in EPA and DHA (see Table 3.5, page 56). The feeding of zooplankton using a mixed diet of PB grown in POME such as POME-PD1 and POME-B1, a EPA and ARA-rich PB (see Table 3.5, page 56) can further increase the survival and growth of larval fish and prawns.

e) Research to improve the survival and growth rate of marble goby fingerlings (> 30 dph)

The survival and growth rate of post-30 dph marble goby fingerlings may be improved by manipulating their nutritional requirement, type of feed (e.g. *Moina* sp. and chironomid larvae fed with POME-PD1; pellet based POME-PD1) and culture conditions. The present study demonstrates the slow growth of marble goby fingerlings in tank culture (Figure 8.4) although they were given highly nutritious feed like bloodworms which contain ca. 52% n-3 HUFA (Lytle et al., 1990) and 56% protein (Sugden, 1973). The suggested quantitative dietary protein level for carnivorous fingerlings weighing 0.5 to 1.0 g is 49%, while for juvenile fish of 10 to 50 g is 47% (FAO, 1987). Further, a diet containing protein level of 40% was reported as the optimal amount for enhancing the growth of tropical carnivorous fish cultured in clear water such as fingerlings of snakehead (*Channa striata*) (Samantaray & Mohantly, 1997) and Asian catfish (*Clarias macrocephalus*) (Evangelista et al., 2005). However, the feeding of marble goby fingerlings with pellets containing 42% protein resulted in low growth (initial size = 4.62 ± 0.31 cm; final size = 4.66 ± 0.30 cm, n = 5) as compared to those fed with bloodworms (initial size = 4.74 ± 0.32 cm; final size = 5.74 ± 0.43 cm, n = 5) after the end of rearing period of 24 days, although no mortality was recorded in both treatments (unpublished data).

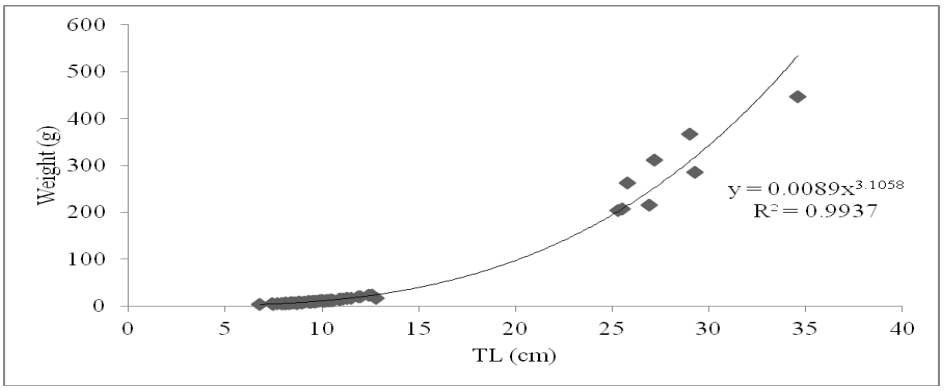


Figure 8.4 Weight versus Total Length (TL) of marble goby juveniles fed with bloodworms, live koi and cockle (this study, unreported)

In a large scale production, it is ideal to rear the marble goby in ponds after they reach a certain age. For instance, fish with a body weight of 80 to 200 g and reared in ponds reached marketable size of 400 - 500 g in a period of 5 to 8 months (Jatuporn, 2007). On the other hand, the rearing of marble goby larvae to adults in earthen ponds could result in low survival (25 - 50%) (Panu et al., 1984). This could be due to improper handling and collection, stress and predators. Hence, further research on survival and growth of post-30 dph marble goby fingerlings is justified to increase its commercial production and viability.

8.6 Conclusion

The findings of the present study can be considered as a big step towards large scale culture of POME-grown bacteria (POME-PD1), rotifers and larval marble goby. The study has fulfilled the overall objective and all the five specific objectives of the component study. The main finding is that POME-PD1 supports rotifer production and significantly improves the survival and growth of larval marble goby (overall objective) as compared to previous studies. The best species of phototrophic bacteria (PB), *Rhodovulum sulfidophilum* (PD1) grown in POME provides adequate DHA and EPA that are crucial for larval survival (specific objective 1), thus supporting the hypothesis that the synthetic medium-grown PB lacks EFA or their precursors for survival of larval fish. PD1 can be mass cultured using POME as a substrate, in any type of reactor (specific objective 2), and its unsettled form (cPOME-PD1) wholly supports the mass production of rotifers (specific objective 3). The rotifers and/or *Artemia* nauplii fed with POME-PD1 significantly improve the survival and growth of newly-hatched marble goby larvae (1 – 30 dph) in 5 ppt salinity and grey coloured tank (specific objective 4 and 5). This finding does not totally accept the hypothesis that the darker coloured tank is a better culture vessel than the lighter one for marble goby larvae. The settled form of

bacteria (bPOME-PD1) is inferior to cPOME-PD1 in mass rotifer production although both forms when respectively given to rotifers and/or *Artemia* nauplii prior to larval feeding are equally good in sustaining larval fish survival and growth. This research has yielded a cheap feed product (POME-PD1) that not only benefits the aquaculture industry but also the palm oil industry by removing the pollutants in POME. Indirectly, an agroindustrial waste has been converted to wealth.

REFERENCES

Abed, G.R., & Zeng, C.S. (2005). Effects of tank colour on larval survival and development of mud crab *Scylla serrata* (Forskål). *Aquaculture Research*, 36, 1112-1119.

Ackman, R.G. (1994). Animal and marine lipids. In B.S. Kamel & Y. Yakuda (Eds.), *Technological advances in improved and alternate sources of lipids* (pp. 292-328). Chapman and Hall: Blackie Acadamic and Professional.

Agh, N., & Sorgeloos, P. (2005). *Handbook of protocols and guidelines for culture and enrichment of live food for use in larviculture*. Ghent, Belgium: University of Ghent.

Ahmad, A.D., Ismail, S., & Bhatia, S. (2003). Water recycling from palm oil mill effluent (POME) using membrane technology. *Desalination*, 157, 87-95.

Alessandro, M., Mario, P.F.C., Giancarlo, C., & Ruggero, G. (1999). *Manual on Hatchery Production of Seabass and Gilthead Seabream, Volume 1* (pp. 1-205). Rome: Food and Agriculture Organization of the United Nations.

Ali, A.B. (1999). Aspects of the reproductive biology of female snakehead (*Channa striata* Bloch) obtained from irrigated rice agroecosystem, Malaysia. *Hydrobiologia*, 411, 71-77.

Alias, Z., & Tan, I.K.P. (2005). Isolation of palm oil-utilising polyhydroxyalkanoate (PHA)-producing bacteria by an enrichment technique. *Bioresource Technology,* 96, 1229-1234.

Alzieu, C. (1990). Water – the medium for culture. In G. Barnabé (Ed.), *Aquaculture volume 1* (pp. 37-196). West Sussex England: Ellis Harwood.

Amornsakun, T., Sriwatana, W., & Chamnanwech, U. (2001). Some aspects in early life stage of sand goby, *Oxyeleotris marmoratus* larvae. 6th *Asian Fisheries Forum Book of Abstract* (pp. 7).

Amornsakun, T., Sriwatana, W., & Chamnanwech, U. (2002). Some aspects in early life stage of sand goby, *Oxyeleotris marmoratus* larvae. *Songklanakarin Journal Science and Technology*, 24, 611-619.

Amornsakun, T., Sriwatana, W., & Chamnanwech, U. (2003). The culture of sand goby, *Oxyeleotris marmoratus* I: Feed and feeding scheme of larvae and juveniles. *Songklanakarin Journal of Science and Technology*, 25(3), 367-371.

Andrade, A.D., Rubira, A.F., Matsushita, M., & Souza, N.E. (2003). n-3 fatty acids in freshwater fish from South Brazil. *Turkish Journal of Biology*, 27, 145-148.

Anonymous (2008). Nutritional profile/proximate analysis (% of dry weight from algae), Retrieved 1 February 2008, from http://www.reed-mariculture.com/support_algae_nutritional_proximate_profile.html.

Association of Official Analytical Chemists (AOAC) (1995). *Official methods of analysis of AOAC international, 2 volumes* (16th ed.). Arlington, VA, USA: Association of Analytical Communities.

Atif, A.A.Y., Fakhru'l-Razi, A., Ngan, M.A., Morimoto, M., Iyuke, S.E., & Veziroglu, N.T. (2005). Fed batch production of hydrogen from palm oil mill effluent using anaerobic microflora. *International Journal of Hydrogen Energy*, 30, 1393-1397.

Azad, S.A. (2004). Growth and production of phototrophic bacterium *Rhodovulum sulfidophilum* and its potential uses as an aquaculture feed. Unpublished PhD's thesis, Institute of Postgraduate Studies, University of Malaya, Kuala Lumpur.

Azad, S.A., Chong, V.C., & Vikineswary, S. (2002). Phototrophic bacteria as feed supplement for rearing *Penaeus monodon* larvae. *Journal of World Mariculture Society*, 33(2), 158-168.

Azad, S.A., Chong, V.C., Vikineswary, S., & Ramachandran, K.B. (2001). Potential of wastegrown phototrophic bacteria as a feed ingredient in aquafeeds. *Journal of Bioscience*, 12(2), 1-11.

Azad, S.A., Vikineswary, S., Chong, V.C., & Ramachandran, K.B. (2003). *Rhodovulum sulfidophilum* in the treatment and utilization of sardine processing wastewater. *Letters in Applied Microbiology*, 38, 13-18.

Azhari, S.B., Lim, S.H., Mohd Zulkhairi, M.Y., Nor' Aini, A.R., Umi, K.M.S., Mohd Ali, H., Minato, W., Kenji, S., & Yoshihito, S. (2010). Effect of palm oil mill effluent (POME) anaerobic sludge from 500 m^3 of closed anaerobic methane digested tank on pressed-shredded empty fruit bunch (EFB) composting process. *African Journal of Biotechnology*, 16(9), 2427-2436.

Baer, K.N., & Goulden, C.E. (1998). Evaluation of a high-hardness COMBO medium and frozen alage for *Daphnia magna*. *Ecotoxicology and Environmental Safety*, 39, 201-206.

Banerjee, S., Azad, S.A., Vikineswary, S., Selvaraj, O.S., & Mukherjee, T.K. (1999). Phototrophic bacteria as fish feed supplement. *Asian Australiasia Journal of Animal Science*, 13(7), 991-994.

Bell, J.G., McEvoy, L.A., Estevez, A., Shields, R.J., & Sargent, J.R. (2003). Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture*, 227, 211-220.

Bell, J.G., & Sargent, J.R. (2003). Arachidonic acid in aquaculture feeds: current status and future opportunities. *Aquaculture*, 218, 491-499.

Bell, M.V., Batty, R., Navarro, J.C., Sargent, J.R., & Dick, J.R. (1995). Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). *Lipids*, 30, 443-449.

Bessonart, M., Izquirerdo, J.S., Salhi, M., Hernandex-Cruz, M.M., Gonzalez, M.M., & Fenandez-Palacios, H. (1999). Effect of dietary arachidonic acid levels on growth and fatty acid composition of gillhead sea bream (*Sparus aurata* L.) larvae. *Aquaculture*, 179, 265-275.

Biebl, H., & Pfennig, N. (1981). Isolation of members of the family *Rhodospirillaceae*. In M.P. Starr, H. Stolp, H.G. Truper, A. Balows, H.G. Schlegel (Eds.), *The prokaryotes*. New York: Springer, Berlin, Heidelberg.

Biswas, J.K., Sarkar, D., Chakraborty, P., Bhakta, J.N., & Jana, B.B. (2006). Density dependent ambient ammonium as the key factor for optimization of stocking density of common carp in small holding tanks. *Aquaculture*, 261, 952-959.

Blaxter, J.H.S. (1968). Light intensity, vision, and feeding in young plaice. *Journal of Experimental Marine Biology and Ecology*, 2, 293-307.

Blaxter, J.H.S. (1986). Development of sense organs and behaviour of teleost larvae with special reference to feeding and predator avoidance. *Transactions of the American Fisheries Society*, 115, 98-114.

Boonyaratpalin, M. (1997). Nutrient requirements of marine food fish cultured in Southeast Asia. *Aquaculture*, 151, 283-313.

Bradner, J., & McRobert, S.P. (2001). Background colouration influences body colour segregation in mollies. *Journal of Fish Biology*, 59, 673-681.

Brett, J.R. (1979). Environmental factors and growth. In W.S. Hoar, D.J. Randall & J.R. Brett (Eds.), *Fish physiology* (pp. 599-675). New York: Academic Press.

Britton, G., & Young, A.J. (1993). Methods for the isolation and analysis of carotenoids. In A.Young & G. Britton (Eds.), *Carotenoids in photosynthetic* (pp. 409-457). London: Chapman and Hall.

Campbell, R.E., Lilley, J.H., Taukhid, Panyawachira, V., & Kanchanakhan, S. (2001). In vitro screening of novel treatments for Aphanomyces invadans. *Aquaculture Research*, 32(3), 223-233.

Castell, J.D., Bell, J.G., Tocher, D.R., & Sargent, J.R. (1994). Effects of purified diets containing different combinations of arachidonic and docosahexaenoic acid on survival, growth and fatty acid composition of juvenile turbot (*Scophthalmus maximus*). *Aquaculture*, 128, 315-333.

Castell, J.D., Blair, T., Neil, S., Howes, K., Mercer, S., Reid, J., Young-Lai, W., Gullison, B., Dhert, P., & Sorgeloos, P. (2001). The effect of different HUFA enrichment emulsions on the nutritional value of rotifers (*Brachionus plicatilis*) to larval haddock (*Melanogrammus aeglefinus*). In Hendry C.I., Van Stappen G., Wille M. & Sorgeloos P. (Eds.), *Larvi 01 – Fish and Shellfish Larviculture Symposium European Aquaculture Society* (pp. 111-114). Oostende, Belgium: Special Publication, vol. 30.

CFRDB (2006). Culture of "pla boo sai" (*Oxyeleotris marmorata*) (in Thai). *Technical leaflet of Aquaculture: Coastal Fisheries Research and Development Bureu (CFRDB)*, 1-2.

Cheah, S.H., Lam, S.Y., & Ang, K.J. (1991). Preliminary report on induced spawning of the marble goby, *Oxyeleotris marmoratus* in Malaysia. *Recent Innovations in the Animal and Animal Product Industry* (pp. 111-114). Serdang: MSAP.

Cheah, S.H., Senoo, S., Lam, S.Y., & Ang, K.J. (1994). Aquaculture of a high value freshwater fish in Malaysia: The marble or sand goby (*Oxyeleotris marmoratus*, Bleeker). *Naga – The ICLAM Quarterly* (pp. 22-25).

Chew, S.F., Tng, Y.M.Y., Wee, L.J.N., Jonathan, M.W., & Ip, Y.K. (2009). Nitrogen metabolism and branchial osmoregulatory acclimation in the juvenile marble goby, *Oxyeleotris marmoratus*, exposed to seawater. *Comparative Biochemistry and Physiology*, part A 154, 360-369.

Chow, M.C., & Ho, C.C. (2002). Chemical composition of oil droplets from palm oil mill sludge. *Journal of Oil Palm Research*, 14(1), 25-34.

Copeman, L.A., & Parrish, C.C. (2004). Lipids classes, fatty acids, and sterols in seafood from Gilbert Bay, Southern Labrador. *Journal of Agricultural and Food Chemistry*, 52, 4872-4881.

Cortney, L.O., Eric, J.C., & Adelaide, R. (2009). Choosing an appropriate live feed for larviculture of marine fish. University of Florida, *IFAS Extension*, FA167.

Coutteau, P., & Sorgeloos, P. (1992). The use of algal substitutes and the requirement for live algae in the hatchery and nursery of bivalve molluscs: an international survey. *Journal of Shellfish Research*, 11, 467-476.

Coutteau, P., & Sorgeloos, P. (1997). Manipulation of dietary lipids, fatty acids and vitamins in zooplankton cultures. *Freshwater Biology*, 38, 501–512.

Coves, D., Audineau, P., & Nicolas, J.L. (1990). Rotifers - rearing technology. In G. Barnabé (Ed.), *Aquaculture, volume 1* (pp. 232-245). West Sussex England: Ellis Harwood.

Cowey, C.B., & Sargent, J.R. (1972). Fish nutrition. *Advances in Marine Biology*, 10, 383-492.

Csengeri, I., Farkas, T., Majoros, F., Olah, J., & Szalay, M. (1978). Effects of feeds on the fatty acid composition of carp (*Cyprinus carpio* L.). *Aquaculture Hungarica (Szarva)*, 1, 24-34.

Cui, J.J., Ding, M.L., Sun, W.L., Liu, H., Wand, D.B., Yu, H.R., & Sun, L.F. (1997). The application of photosynthetic bacteria to the seed production of *Penaeus chinensis*. *Journal of Ocean University of Qingdao* (in Chinese), 27, 191-194.

David, M.A., Allen, P.D., & Paul, M.G. (1994). Removing heavy metals from wastewater. *Engineering Research Center Report*, University of Maryland, 1-21.

Department of Fisheries Malaysia (2009). Annual Fisheries Statistics 2009 Volume 1. Jabatan perikanan Malaysia.

De-Santis, C., & Dean, R.J. (2007). Candidate growth genes in finfish - Where should we be looking. *Aquaculture*, 272, 22-38.

Dhert, P. (1996). Rotifers. In P. Lavens & P. Sorgeloos (Eds.), *Manual on the Production and Use of Live Food for Aquaculture* (pp. 61-99). Food and Agriculture

Organisation of the United Nations, Fisheries and Aquaculture Department: FAO Fisheries Technical Paper, No. 361.

Dhert, P., Lavens, P., Duray, M., & Sorgeloos, P. (1990). Improved larval survival at metamorphosis of Asian seabass (*Lates calcarifer*) using 3 HUFA-enriched live food. *Aquaculture*, 128, 315-333.

Dhert, P., Rombaut, G., Suantika, G., & Sorgeloos, P. (2001). Advancement of rotifer culture and manipulation techniques in Europe. *Aquaculture*, 200, 129-146.

Dhert, P., Sorgeloos, P., & Devresse, B. (1993). Contributions towards a specific DHA enrichment in the live food *Brachionus plicatilis* and *Artemia* sp. In H. Reinertsen, L.A. Dahle, L. Jorgensen & K. Tvinnereim (Eds.), Fish Farming Technology (pp. 109-115). Balkema, Rotterdam, Netherlands.

Dhert, P., Suantika, G., De Wolf, T., Okechi, J.K., Bonaldo, A., & Sorgeloos, P. (2000). The use of ozone in a high density rotifer recirculation system. In R, Flos & L, Creswell (Eds.), *Responsible Aquaculture in the New Millenium* (pp. 182). AQUA 2000, Nice, France: European Aquaculture Society, Special Publication, 28.

Dou, S., Masuda, R., Tanaka, M., & Tsukamoto, K. (2002). Feeding resumption, morphological changes and mortality during starvation in Japanese flounderlarvae. *Journal of Fish Biology*, 60, 1363-1380.

Downing, G., & Litvak, M.K. (1999). The effect of photoperiod, tank colour and light intensity on growth of larval haddock. *Aquaculture International*, 7, 369-382.

Duray, M.M., Estudillo, C.B., & Alpasan, L.G. (1997). Larval rearing of the grouper *Epinephelus suillus* under laboratory conditions. *Aquaculture*, 150, 63-76.

Emilie, H., Robert, S.N.M.M., Agbohessi, T.P., Michaël, V., Charles, M., & Patrick, K. (2010). Egg and larval quality, and egg fatty acid composition of Eurasian perch breeders (*Perca fluviatilis*) fed different dietary DHA/EPA/AA ratios. *Aquaculture Research*, 41, e53-e61.

Evangelista, A.D., Fortes, N.R., & Santiago, C.B. (2005). Composition of some live organisms and artificial diet as feed for Asian catfish *Clarias macrocephalus* (Gunther) larvae. *Journal of Applied Ichthyology*, 21, 437-443.

Fabiano, L.T., Paulo, C.A., & Ronaldo, C. (1999). The use of microorganisms as food source for *Penaeus paulensis* larvae. *Aquaculture*, 174, 139-153.

FAO (1980). *Amino acid content of foods and biological data on proteins*. Rome, Italy: FAO Nutritional Studies No. 24.

FAO (1987). In Food and Agriculture Organisation of the United Nations (Ed.). *The nutrition and feeding of farmed fish and shrimp - A training manual 1. The essential nutrients*. GCP/RLA/075/ITA, Rome, Italy: FAO.

FAO (2010). *The state of world fisheries and aquaculture*. Rome: FAO Fisheries and Aquaculture Department, Food and Agriculture Organization of the United Nations.

Farhat, J., & Abdul Shakoor, C. (2011). Chemical compositions and fatty acid profiles of three freshwater fish species. *Food Chemistry*, 125, 991-996.

Ferreira, P.P., Santos, P., Carvalho, A.P., Morais, S. & Narciso, L. (2003). Effect of an experimental microparticulate diet on the growth, survival and fatty acid profile of gilthead seabream (*Sparus aurata* L.) larvae. *Aquaculture International*, 11, 491-504.

Firsow, N.N., & Drews, G. (1977). Differentiation of the intracytoplasmic membrane of *Rhodopseudomonas palustris* induced by variations of oxygen partial pressure or light intensity. *Archives of Microbiology*, 115, 299-306.

Foo, K.Y., & Hameed, B.H. (2010). Insight into the applications of palm oil mill effluent: a renewable utilization of the industrial agricultural waste. *Renewable and Sustainable Energy Reviews*, 14, 1445-1452.

Forsgren, E. (1997). Female sand gobies prefer good fathers over dominant males. *Proceedings of the Royal Society of London, Series B*, 264, 1283-1286.

Fu, Y., Hada, H., Yamashita, T., Yoshida, Y., & Hino, A. (1997). Development of continuous culture system for stable mass production of the marine rotifer *Brachionus*. *Hydrobiologia*, 358, 145-151.

Fukusho, K. (1983). Present status and problems in culture of the rotifer *Bachionus plicatilis* for fry production of marine fishes in Japan. *Symposium International De Aquacultura* held in September 1983 in Coquimbo, Chile (pp. 361-374).

Fukusho, K. (1989). Biology and mass production of the rotifer, *Brachionus plicatilis*. *International Journal of Aquaculture and Fisheries Technology*, 1, 232-240.

Galid, R.S. (2003). *Investment opportunities in aquaculture industry in Sabah, Malaysia*. Paper presented at the 7th BIMPEAGA working group on fisheries cooperation meeting, Palawan, Philippines.

Galkovskaja, G.A. (1987). Planktonic rotifers and temperature. *Hydrobiologia*, 147, 307-317.

Gatesoupe, F.J. (1999). The use of probiotics in aquaculture. *Aquaculture*, 180, 147-165.

Gerhard, G. (1986). *Bacterial metabolism* (2nd ed.). Springer.

Gest, H., & Favinger, J.L. (1983). *Heliobacterium chlorum*, an anoxygenix brownish-green photosynthetic bacterium containing a "new" form of bacterio-chlorophyll. *Archives of Microbiology*, 136, 11-16.

Getha, K. (1995). Growth and production of the phototrophic bacterium *Rhodopseudomonas palustris* strain B1 in sago starch processing wastewater. Unpublished master of biotechnology's thesis, Institute of Advanced Studies, University of Malaya, Kuala Lumpur, Malaysia.

Getha, K., Chong, V.C., & Vikineswary, S. (1998b). Potential use of the phototrophic bacterium *Rhodopseudomonas palustris*, as in aquaculture feed. *Asian Fisheries Science*, 10, 223-232.

Getha, K., Vikineswary, S., & Chong, V.C. (1998a). Isolation and growth of the phototrophic bacterium *Rhodopseudomonas palustris* strain B1 in sago-starch processing wastewater. *World Journal of Microbiology and Biotechnology*, 14, 505-511.

Gilbert, J.J., & Jack, J.D. (1993). Rotifers as predators on small ciliates. *Hydrobiologia*, 255/256, 247-253.

Girin, M., & Devauchele, B. (1974). Production du rotifóre *Brachionus plicatilis O.F.* Moller en 6levage mixte avec le copópode tisbe farcata (Baird). "Meeting I.C.E.G. work. Group Maricult". *Act. Coll. CNEXO*, 1, 87-99.

Granvil, D.T. (2000). *Artemia* production for marine larval fish culture. *Southern Regional Aquaculture Center Publication*, 702.

Gurmit, S., Lim, K.H., Teo, L., & Lee, D.K. (1999). Oil palm and the environment a Malaysian perspective, volume 1. *Malaysian Oil Palm Growers Council*, 253.

Habib, M.A.B., Yusoff, F.M., Phang, S.M., Ang, K.J., & Mohamed, S. (1997). Nutritional values of chironomoid larvae grown in palm oil mill effluent and algal culture. *Aquaculture*, 158, 95-105.

Habib, M.A.B., Yusoff, F.M., Phang, S.M., Kamarudin, M.S., & Mohmed, S. (1998). Chemical characteristics and essential nutrients of agro-industrial effluents in Malaysia. *Asian Fisheries*, 11, 279-286.

HACH water analysis handbook (3rd ed.) (1997). Loveland, Colorado, USA: HACH company.

Hallenbeck, P.L., Lerchen, R., Hessler, P., & Kaplan, S. (1990). Roles of CfxA, CfxB, and external electron acceptors in regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase expression in *Rhodobacter sphaeroides*. *Journal of Bacteriology*, 172, 1736-1748.

Han, K., Geurden, I., & Sorgeloos, P. (2000). Enrichment strategies for *Artemia* using emulsions providing different levels of n-3 highly unsaturated fatty acids. *Aquaculture*, 183, 335-347.

Hansen, T.A. (1974). Sulfide als electronendonor voor Rhodospirillaceae, Unpublished PhD's thesis, University of Groningen, The Netherlands.

Hansen, T.A., & Veldkamp, H. (1973). *Rhodopseudomonas sulfidophila*, nov. spec., a new species of the purple nonsulfur bacteria. *Archives of Microbiology*, 92, 45-58.

Hardjamulia, A., Utami, S., & Suhendra, R. (1988). Effects of salinity on survival and growth of river catfish (*Pangasius sutchi*). *Bulletin Research of Freshwater Fish*, 2, 1-5.

Hartley, C.W.S. (1967). The oil palm. In Longman (pp. 1-36). London: Green and company Ltd.

Head, W.D., Watanabe, W.O., Ellis, S.C., & Ellis, E.P. (1994). Hormone-induced multiple spawning of captive Nassau grouper (*Epinephelus striatus*) broodstock. *Book of Abstracts, World Aquaculture* (pp. 149). LA: New Orleans.

Helland, S.J., Grisdale-Helland, B., & Nerland, S. (1996). A simple method for the measurement of daily feed intake of groups of fish in tanks. *Aquaculture*, 139, 157-163.

Henderson, R.J. (1996). Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. *Archives of Animal Nutrition*, 49, 5-22.

Henderson, R.J., & Sargent, J.R. (1985). Chain-length specificities of mitochondrial and peroxisomal oxidation of fatty acids in livers of rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry and Physiology*, 82B, 79-85.

Henderson, R.J., & Tocher, D.R. (1987). The lipid composition and biochemistry of freshwater fish. *Progress in Lipid Research*, 26, 281-347.

Herbert, R.A. (1976). Isolation and identification of photosynthetic bacteria (Rhodospirillaceae) from Antarctic marine and freshwater sediments. *Journal of Applied Bacteriology*, 41, 75-80.

Hessen, D.O., & Andersen, T. (1990). Bacteria as a source of phosphorus for zooplankton. *Hydrobiologia*, 206, 217-223.

Hillmer, P., & Gest, H. (1977a). H_2 metabolism in the photosynthetic bacterium *Rhodopsuedomonas capsulata*: H_2 production by growing cultures. *Journal of Bacteriology*, 129, 724-731.

Hillmer, P., & Gest, H. (1977b). H_2 metabolism in the photosynthetic bacterium *Rhodopsuedomonas capsulata*: production and utilization of H_2 by resting cell. *Journal of Bacteriology*, 129, 732-739.

Hino, A., Aoki, S., & Ushiro, M. (1997). Nitrogen-flow in the rotifer *Brachionus rotundiformis* and its significance in mass cultures. *Hydrobiologia*, 358, 77-82.

Hinshaw, J.M. (1985). Effects of illumination and prey contrast on survival and growth of larval yellow perch *Perca flavescens*. *Transactions of the American Fisheries Society*, 114, 540-545.

Hirata, H. (1979). Rotifer culture in Japan. In E. Styczynska-Jurewicz., T. Backiel, E. Jaspers, & G. Persoone (Eds.). Cultivation of fish fry and its live food. *European Mariculture Society Special Publication*, 4, 361-375.

Hirata, H. (1980). Culture methods of the marine rotifer, *Brachionus plicatilis*. *Mini Review and Data File of Fisheries Research, Kagoshima University*, 1, 27-46.

Hirata, H., & Nagata, W.D. (1982). Excretion rates and excreted components of the rotifer *Brachionus plicatilis* O.F. Müller in culture. *Memoirs of the Faculty of Fisheries, Kagoshima University*, 31, 161-174.

Hirayama, K. (1987). A consideration of why mass culture of the rotifer *Brachionus plicatilis* with baker's yeast is unstable. *Hydrobiologia*, 147, 269-270.

Hirayama, K., & Nakamura, K. (1976). Fundamental studies on the physiology of rotifer in mass culture - V. Dry *Chlorella* powder as a food for rotifers. *Aquaculture*, 8, 301-307.

Hirayama, K., & Rumengan, I.F.H. (1993). The fecundity patterns of S- and L-types rotifers of *Brachionus plicatilis*. *Hydrobiologia*, 255/256, 153-157.

Hirayama, O., Ando, E., Wamori, K., & Hara, N. (1974). Kougousei saikin *Rhodopseudomonas capsulatus* no shikiso seisei to baiyou jouken (Effect of culture conditions on pigment formation by *Rhodopseudomonas capsulatus*) (in Japanese). *Nippon Nôgeikagaku Kaishi*, 48, 97–104.

Hirotani, H., Ohigashi, H., Kobayashi, M., Koshimizu, K., & Takashi, E. (1991). Inactivation of T5 phage by cis-vaccenic acid and antivirus substances from *Rhodopseudomonas capsulate*, and by unsaturated fatty acids and related alcohols. *FEMS Microbiology Letters*, 77, 13-18.

Hoa, N.P., & Yi, Y. (2007). Prey ingestion and live food selectivity of marble goby (*Oxyeleotris marmorata*) using rice field prawn (*Macrobrachium lanchesteri*) as prey. *Aquaculture*, 273, 443-448.

Hoff, F.H., & Snell, T.W. (1989). *Plankton culture manual* (2nd ed.) (pp. 126). Florida: Florida Aqua Farms.

Hoff, F.H., & Snell, T.W. (1997). *Plankton culture manual* (4th ed.). Dade City, Florida: Florida Aqua Farms.

Hoglund, E., Balm, P.H.M., & Winberg, S. (2002). Behavioural and neuroendocrine effects of environmental background colour and social interaction in Arctic charr (*Salvelinus alpinus*). *Journal of Experimental Biology*, 205, 2535-2543.

Howell, B.R. (1973). Marine fish culture in Britain VIII. A marine rotifer, *Brachionus plicatilis* Muller, and the larvae of the mussel, *Mytilus edulis* L., as foods for larval flatfish. *Journal Du Conseil International Pour L'Exploration De La Mer*, 35, 1-6.

Howell, B.R. (1979). Experiments on the rearing of larval turbot *Scophthalmus maximus* L. *Aquaculture*, 18, 215-225.

Howell, B.R., & Tzoumas, T.S. (1991). The nutritional value of *Artemia* nauplii for larval sole, *Solea solea* (L.) with respect to their (n - 3) HUFA content. *European Aquaculture Society Special Publication*, 15, 63-65.

Ikenoue, H. (1991). Aquaculture in Thailand; sand goby culture (in Japanese). *Yoshoku*, 28(10), 117-119.

Imam, M.M. (1984). Oil palm in Malaysia: Chronology of events. *PORIM Bulletin, Special Issue*, 5-7.

Imhoff, J.F. (1992). Taxonomy, phyologeny, and general ecology of anoxygenix phototrophic bacteria. In N.G. Carr & N.H. Mann (Eds.), *Biotechnology handbook photosynthetic prokaryotes* (pp. 53-92). London & New York: Plenum.

Imhoff, J.F. (1995). Taxonomy, phylogeny, and general ecology of anoxygenic phototrophic bacteria. In R.E. Blankenship, M.T. Madigan & C.E. Bauer (Eds.), *Anoxygenic photosynthetic bacteria* (pp. 1-15). The Netherlands: Kluwer Academic Publisher.

Imhoff, J.F. (2001). True marine and halophilic anoxygenic phototrophic bacteria. *Archives of Microbiology*, 176, 243-254.

Imhoff, J.F., & Imhoff, U.B. (1995). Lipids, quinines and fatty acids of anoxygenic phototrophic bacteria. In R.E. Blankenship, M.T. Madigan & C.E. Bauer (Eds.), *Anoxygenic phototrophic bacteria* (pp. 179-205). Boston, London: Kluwer Academic Publisher.

Imhoff, J.F., & Truper, H.G. (1989). The purple nonsulfur bacteria. In J.T. Staley, M.P. Bryant, N. Pfennig & J.C. Holt (Eds.), *Bergey's manual of systematic bacteriology, volume 3* (pp. 1658-1661). Baltimore: Williams and Wilkins.

Imhoff, J.F., & Truper, H.G. (1992). The genus *Rhodospirillum* and related genera. In A. Balows, H.G. Truper, M. Dworkin, W. Harder & K.H. Schleifer (Eds.), *The prokaryotes* (2nd ed.) (pp. 2141-2152). New York: Springer-Verlag.

Ina, K., Ryogi, Y., & Higashi, K. (1979). Color sensitivity of red sea bream, *Pagrus major*. *Bulletin of the Japanese Society for the Science of Fish*, 45(1), 1-5.

Industrial Processes and the Environment (1999). *Crude palm oil industry (Handbook No. 3)*. Malaysia: Department of Environment, Ministry of Science, Technology and the Environment.

Inger, R.F., & Chin, P.K. (2002). The freshwater fishers of North Borneo (2nd ed.) (pp. 268). Kota Kinabalu: Natural History Publication (Borneo).

Izquierdo, M.S., Socorro, J., Arantzamendi, L., & Hernández-Cruz, C.M. (2000). Recent advances in lipid nutrition in fish larvae. *Fish Physiology and Biochemistry*, 22, 97-107.

Izu, K., Nakajima, F., Yamamoto, K., & Kurisu, F. (2001). Aeration conditions affecting growth of purple nonsulfur bacteria in organic wastewater treatment process. *Systematic and Applied Microbiology*, 24, 294-302.

James, C.M., & Rezeq, T.A. (1989). Production and nutritional quality of two small-sized strains of the rotifer *Brachionus plicatilis. Journal of the World Aquaculture Society*, 20, 1-15.

Jatuporn, B. (2007). The nutrition and feeding of a native Thai species, the marble goby (*Oxyeleotris marmoratus*), Involving On-farm and Experimental Studies. Unpublished PhD's thesis, University of Stirling.

Jauncey, K. (1982). The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapias (*Sarotherodon mossambicus*). *Aquaculture*, 27, 43-54.

Jayaram, K.C. (1999). *The freshwater fishes of the Indian Region*. India: Narendra Publishing House Delhi-110006.

Jennifer, M.C., & Stephen, C.B. (2009). Jaw malformation in striped trumpeter *Latris lineate* larvae linked to walling behaviour and tank colour. *Aquaculture*, 289(3-4), 274-282.

Jensen, S.L., & Jensen, A. (1971). Quantitative determination of carotenoids in photosynthetic tissues. In A.S. Pietro (Ed.), *Methods in enzymology, volume 23* (pp. 586-602). New York, USA: Academic Press.

Jobling, M., & Baardvik, B.M. (1994). The influence of environmental manipulation on inter and intra-individual variation in food acquisition and growth performance of Arctic charr, *Salvelinus alpines*. *Journal of Fish Biology*, 44, 1069-1087.

Jobling, M., Koskela, J., & Savolainen. (1998). Influence of dietary fat level and increased adiposity on growth and fat deposition in rainbow trout *Onrcorhynchus mykiss* (Walbaum). *Aquaculture Research*, 29, 601-607.

John, S., Gordon, B., Lesley, M., Douglas, T., & Alicia, E. (1999). Recent developments in the essential fatty acid nutrition in fish. *Aquaculture*, 177, 191-199.

Jones, J.C., & Reynolds, J.D. (1999). Oxygen and the trade-off between egg ventilation and brood protection in the common goby. *Behaviour*, 136, 819-832.

Jonna, R., & Weinheimer, M. (2003). "Eleotridae. Retrieved 2 March 2011, from http://animaldiversity.uumz.umich.edu/site/accounts/information/Eleotridae.html

Jose, L.B., Ignacio, D.B., Imanol, R.Z., David, C., Daniel, V., & Jose, L.M. (2006). The role of probiotics in aquaculture. *Veterinary Microbiology*, 114, 173-186.

Jow, L., Chew, S., Lim, C., Anderson, P., & Ip, Y. (1999). The marble goby *Oxyeleotris marmoratus* activities hepatic glutamine synthetase and detoxifies ammonia to glutamine during air exposure. *Journal of Experimental Biology*, 202, 237-245.

Kanazawa, A. (1997). Effects of docosahexaenoic acid and phospholipids on stress tolerance of fish. *Aquaculture*, 155, 129-134.

Kantachote, D., Torpee, S., & Umsakul, K. (2005). The potential use of anoxygenic phototrophic bacteria for treating latex rubber sheet wastewater. *Electronic Journal of Biotechnology*, 8, 314-323.

Kim, J.K., & Lee, B.K. (2000). Mass production of *Rhodopseudomonas palustris* as diet for aquaculture. *Aquacultural Engineering*, 23, 281-293.

Kim, J.S., Ito, K., & Takahashi, H. (1982). Production of molecular hydrogen in outdoor batch cultures of *Rhodopseudomonas sphaeroides*. *Agricultural and Biological Chemistry*, 46, 937-941.

Kiorboe, T., Mohlenberg, F., & Hamburger, V. (1985). Bioenergetics of the planktonic copepod *Acartia tonsa*: relation between feeding, egg production and respiration and composition of specific dynamic action. *Marine Ecology*, 26, 85-97.

Kobayashi, M. (1995). Waste remediation and treatment using anoxygenic phototrophic bacteria. In R.E. Blenkenship, M.T. Madigan & C.E. Bauer (Eds.). *Anoxygenic photosynthetic bacteria* (pp. 1272-1274). Dordrecht: Kluwer Academic.

Kobayashi, M., & Kobayashi, M. (1995). Waste remediation and treatment using anoxygenic phototrophic bacteria. In R.E. Blankenship, M.T. Madigan & C.E. Bauer

(Eds.), *Anoxygenic photosynthetic bacteria* (pp. 1269-1282). Dordrecht: Kluwer Academic Publisher.

Kobayashi, M., & Kobayashi, M. (2001). Roles of phototrophic bacteria and their utilization. In H. Kojima & Y.K. Lee (Eds.), *Photosynthetic microorganism in environmental biotechnology* (pp. 11-26). Hong Kong: Springer-Verlag.

Kobayashi, M., & Kurata, S. (1978). The mass culture and cell utilization of photosynthetic bacteria. *Process Biochemistry*, 9(12), 27-30.

Kohler, C.C., Sheehan, R.J., Habicht, C., Malison, J.A., & Kayes, T.B. (1994). Habituation to captivity and controlled spawning of white bass. *Transactions of the American Fisheries Society*, 123, 964-974.

Kok, L.W. (1982). Snakehead - their biology and culture. In Anonymous, *Recent advance in aquaculture* (pp. 179-214). London: Croom Helm Ltd.

Kondratieva, E.N., & Krasil'nikova, E.N. (1981). Reduction of nitrates by purple bacteria under various conditions of growth. *Mikrobiologiya*, 50, 1066-1070.

Konrad, D., & Bardega, R. (1984). Mouth size and predicted food size preferences of larvae of three cyprinid fish species. *Aquaculture*, 40(1), 41-46.

Kottelat, M. (2001). Fishes of Laos (pp. 198). Colombo 5, Sri Lanka: Wildlife Heritage Trust Publications.

Kottelat, M., Whitten, A.J., Kartikasari, S.N., & Wirjoatmodjo, S. (1993). Freshwater Fisheries of Western Indonesia and Sulaiwesi (pp. 221). Jakarta: Periplus.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P., & Tandler, A. (2001). The effect of dietary arachidonic acid (20:4*n*−6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. *Aquaculture*, 193(1-2), 107-122.

Krebs, C.J. (1985). Ecology. The experimental analysis of distribution and abundance (pp. 789). New York: Harper and Row.

Krishna, C., & Young, M.M. (2004). Single-cell protein. In A. Pandey (Ed), *Concise Encyclopedia of Bioresource Technology*. United States of America: The Haworth Press, Inc.

Lavens, P., & Sorgeloos, P. (Eds.) (1996). *Manual on the Production and Use of Live Food for Aquaculture* (pp. 9-100). Rome, Food and Agriculture Organization of the United Nations: FAO Fisheries Technical Paper, No. 361.

Leatherland, F.J., Reddy, K.P., Yong, N.A., Leatherland, A. & Lam, T.J. (1990). Hepatic 5' monodeiodinase activity in teleost in vitro: A survey of thirty-three species. *Physiology and Biochemistry*, 8, 1-10.

Léger, P., Bieber, G.F., & Sorgeloos, P. (1985). International study on *Artemia* XXXIII. Promising results in larval rearing of *Penaeus stylirostris* using a prepared diet as algal substitute and for *Artemia* enrichment. *Journal of the World Mariculture Society*, 16, 354-367.

Léger, P., Naessens-Foucquaert, E., & Sorgeloos, P. (1987). International study on *Artemia* XXXV. Techniques to manipulate the fatty acid profile in *Artemia* nauplii, and the effect on its nutritional effectiveness for the marine crustacean *Mysidopsis bahia* (M). In P. Sorgeloos, D.A. Bengston, W., Decleir & E. Jaspers (Eds.), *Artemia Research and its Applications. 3. Ecology, Culturing, Use in Aquaculture* (pp. 411-424). Wetteren, Belgium, Universal Press.

Lin, C.K., & Kaewpaitoon, K. (2000). An overview of freshwater cage culture in Thailand. In Lin C.K. (Ed.), *Cage Aquaculture in Asia*: *Proceedings of the First International Symposium on Cage aquaculture in Asia* (pp. 253-257).

Lubzens, E. (1987). Raising rotifers for use in aquaculture. *Hydrobiologia*, 147, 245-255.

Lubzens, E., Gibson, O., Zmora, O., & Sukenik, A. (1995). Potential advantages of frozen algae (*Nannochloropsis* sp.) for rotifer (*Brachionus plicatilis*) culture. *Aquaculture*, 133, 295-309.

Lubzens, E., Marko, A., & Tietz, A. (1985a). De novo synthesis of fatty acids in the rotifers, *Brachionus plicatilis*. *Aquaculture*, 47, 27-37.

Lubzens, E., Minkoff, G., & Marom, S. (1985b). Salinity dependence of sexual and asexual reproduction in the rotifer *Brachionus plicatilis*. *Marine Biology*, 85, 123-126.

Lubzens, E., Tandler, A., & Minkoff, G. (1989). Rotifers as food in aquaculture. *Hydrobiologia*, 186/187, 387-400.

Luong, V.C., Yang, Y., & Lin, C.K. (2005). Cove culture of marble goby (*Oxyeleotris marmorata* Bleeker) and carps in Tri An Reservoir of Vietnam. *Aquaculture*, 244, 97-107.

Lytle, J.S., Lytl, T.F., & Ogle, J. (1990). Polyunsaturated fatty acid profiles as a comparative tool in assessing maturation diets of *Penaeus vannamei*. *Aquaculture*, 89, 287-299.

Ma, A.N. (2000). Environmental management for the palm oil industry. *Palm Oil Development*, 30, 1-10.

Ma, A.N., Chow, C.S., John, C.K., Ibrahim, A., & Isa, Z. (2001). Palm oil mill effluent - a survey. In *Proceeding PORIM Regional Workshop on Palm Oil Mill Technology and Effluent Treatment* (pp. 233-269). Serdang, Malaysia: Palm Oil Research Institute of Malaysia (PORIM).

Madigan, M.T. (1999). Isolation and characterization of psychrophilic purple bacteria from Antarctica. In G.A. Peschek, W. Loffelhardt & G. Schmetterer (Eds.), *The phototrophic prokaryotes* (pp. 699-706). New York, Boston, Dordrecht, London, Moscow: Kluwer Academic/Plenum Publishers.

Madigan, M.T., Cox, J.C., & Gest, H. (1980). Physiology of dark fermentative growth of *Rhodopseudomonas capsulate*. *Journal of Bacteriology*, 142(3), 908-915.

Madigan, M.T., & Gest, H. (1979). Growth of the photosynthetic bacterium *Rhodopseudomonas capsulate* chemoautotrophically in darkness with H_2 as the energy source. *Journal of Bacteriology*, 137, 524-530.

Madigan, M.T., & Gest, H. (1988). Selective enrichment of isolation of *Rhodopseudomonas palustris* using *trans*-cinnamic acid as sole carbon source. *FEMS Microbiology Letters*, 53(1), 53-58.

Madigan, M.T., Martinko, J.M., & Parker, J. (2000). *Brock biology of microorganism*. (9th ed.). Upper Saddle River, New Jersey: Prentice Hall International, Inc.

Madukasi, E.I., Dai, X., He, C., & Zhou, J. (2010). Potentials of phototrophic bacteria in treating pharmaceutical wastewater. *International Journal of Environmental Science and Technology*, 7(1), 165-174.

Maheswari, S. (1997). Optimization of growth and immobilization of *Rhodopsuedomonas palustris* strain B1 for the utilization of sago starch processing

wastewater. Unpublished master of biotechnology's thesis, Institute of Advanced Studies, University of Malaya, Kuala Lumpur, Malaysia.

Maheswari, S., Vikineswary, S., Shaliza, I., & Sastry, C.A. (1998). Photoutilisation of sago effluent by immobilized *Rhodopseudomonas palustris* strain B1. In Jose, C., & Rasyad, A. (Eds.). *Proceedings of 6th Sago Symposium 'Sago: The Future Source of Food and Feed*'. Indonesia: Riau University Training Centre.

Mahmoud, I.A.H., & Layla, H. (2003). Experiments on the control of anchor worm (*Lernaea cyprinacea*). *Aquaculture*, 2, 45-51.

Malaysian Palm Oil Board - MPOB (2010). Overview of the Malaysian oil palm industry 2009. Ministry of Plantation Industries and Commodities Malaysia.

Manop, T. (1984). Artificial breeding of sand goby. In Anonymous (Ed.), *Artificial Breeding of Economic Fish Species*. Department of Fisheries Technical Paper, Bangkok, Thailand.

Martin-Robichaud, D.J., & Peterson, R.H. (1998). Effects of light intensity, tank colour and photoperiod on swimbladder inflation success in larval striped bass, *Morone saxatilis* (Walbaum). *Aquaculture Research*, 29, 539-547.

Masaya, M., Makoto, O., Endang, S.S., Bambang, P.S., Diptarina, M., Lilik, H., & Akihiro, T. (2006). Changes in sperm motility in respond to osmolality/Ca^{2+} in three Indonesian freshwater teleosts: Goby (*Oxyeleotris marmorata*). *Comparative Biochemistry and Physiology, Part A*, 143, 361-367.

Masuda, S., Nagashima, K.V.P., Shimada, K., & Matsuura, K. (2000). Transcriptional control of expression of genes for photosynthetic reaction center and light-harvesting proteins in the purple bacterium *Rhodovulum sulfidophilum*. *Journal of Bacteriology*, 182, 2778-2786.

Matsuda, H., Tsujigado, A., & Yamakawa, T. (1987). Effect of environmental factors on the survival and growth of larvae of stone flounder *Kareius bicolouratus*. *Bulletin of Fisheries Research, Institute of Mie*, 2, 45-50.

McDowall, R.M. (2007). On amphidromy, a distinct form of diadromy in aquatic organisms. *Fish and Fisheries*, 8, 1-13.

McEvoy, L.A., Navarro, J.C., Hontario, F., Amat, F., & Sargent, J.R. (1996). Two novel *Artemia* enrichment diets containing polar lipid. *Aquaculture*, 144, 339-352.

Meragelman, E., Lubzens, E., & Minkoff, G. (1985). A modular system for small-scale mass production of the rotifer *Brachnionus plicatilis*. *Israel Journal of Zoology*, 33, 186-194.

Michael, A.B. (1997). Microalgae for aquaculture: Opportunities and constraints. *Journal of Applied Phycology*, 9, 393-401.

Milan, C., & Sakayu, S. (1999). Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *Journal of Bioscience and Bioengineering*, 87(1), 1-14.

Ministry of Agriculture Malaysia (2000). 'Opportunities in Agriculture' NAP3 - A Comprehensive Approach Towards Agricultural Development in Malaysia. *Persidangan Tahunan MANCID Ke 8 (8th MANCO)* held on 23-24 November, 2000.

Mohsin, A.K.M., & Ambak, M.A. (1983). *Freshwater fishes of Peninsula Malaysia* (pp. 284). Kuala Lumpur: Penerbit Universiti Pertanian Malaysia.

Moksness, E., Kjørsvik, E., & Olsen, Y. (2004). *Culture of cold-water marine fish* (pp. 528). Oxford: Blackwell Publishing.

Monk, J., Puvanendran, V., & Brown, J.A. (2006). Do different light regimes affect the foraging behaviour, growth and survival of larval cod (*Gadus morhua* L.)? *Aquaculture*, 257, 287-293.

Montagnes, D.J.S., Kimmance, S.A., Tsounis, G., & Gumbs, J.C. (2001). Combined effect of temperature and food concentration on the grazing rate of the rotifer *Brachionus plicatilis*. *Marine Biology*, 139, 975-979.

MPOC (Malaysian Palm Oil Council) (2006). Oil palm…tree of life. Retrieved 1 March 2010, from http://www.mpoc.org.my/

Muhammad Darwis, Siti Raehana, M.S., & Senoo, S. (2008). Effects of different salinity levels on growth and survival of marble goby, *Oxyeleotris marmoratus* juvenile. *Aquaculture Science*, 56(3), 433-439.

Muhammad Darwis, Siti Raehana, M.S., Tanaka, M., & Senoo, S. (2009). Effects of different types of feed on growth, survival and digestive enzyme activity of marble goby, *Oxyeleotris marmoratus* juveniles. *Oseanologi dan Limnologi di Indonesia*, 35(1), 1-18.

Naas, K., Huse, I., & Iglesias, J. (1996). Illumination in first feeding tanks for marine fish larvae. *Aquacultural Engineering*, 15, 291-300.

Naas, K.E., Næss, T., & Harboe, T. (1992). Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus* L.). *Aquaculture*, 105, 143-156.

Nasir, M. (1994). Preventive of natural feed using diazinon 60 EC on growth and survival of sand goby, *Oxyeleotris marmoratus* larvae. Unpublished BSc.'s thesis, University of Bogor Agriculture.

Neik, T.S. (2006). The enhancement of growth and survival of ornamental koi (*Cyprinus carpio*) larvae using phototrophic bacteria. Unpublished MBiotech's thesis, Faculty of Science, University of Malaya, Kuala Lumpur.

Nguyen, T.N.T., Nguyen, N.P., Kristof, D., Patrick, S., & Peter, B. (2005). Gnotobiotically grown rotifer *Brachionus plicatilis* sensu strictu as a tool for evaluation of microbial functions and nutritional value of different food types. *Aquaculture*, 253, 421-432.

Nicolás, J.L., Robic, E., & Ansquer, D. (1989). Bacterial flora associated with a trophic chain consisting of microalgae, rotifers and turbot larvae: influence of bacteria on larval survival. *Aquaculture*, 83, 237-248.

Nielsen, N.S., Gittsche, J.R., Holm, J., Xu, X., Mu, H., & Jacobsen, C. (2005). Effect of structured lipids based on fish oil on the growth and fatty acid composition in rainbow trout (*Oncorhychus mukiss*). *Aquaculture*, 250, 411-423.

Nishizawa, Y., Nagai, S., & Aiba, S. (1974). Growth yields of *Rhodopseudomonas sphaeroides* in dark and aerobic chemostat cultures. *Journal of Fermentation Technology*, 52(8), 526-535.

Noor Hazira, B.A.B. (2006). Study on mechanical pretreatment process of palm oil mill effluent (POME). Unpublished degree of bachelor of chemical engineering's thesis, Faculty of Chemical Engineering and Natural Resources, University College of Engineering and Technology Malaysia.

Noparatnaraporn, N., & Nagai, S. (1986). Selection of *Rhodopseudomonas sphaeroides* P47 as a useful source of single cell protein. *Journal of General and Applied Microbiology*, 32, 351-359.

Noparatnaraporn, N., Nishizawa, Y., Hayashi, M., & Nagai, S. (1983). Single cell protein production from cassava starch by *Rhodopseudomonas gelatinosa*. *Journal of Fermentation Technology*, 61, 515-519.

Noparatnaraporn, N., Trakulnaleumsai, S., & Duangsawat, S. (1987). Journal of Science Society of Thailand, 13, 15. Cited in Sasaki, K., Tanaka, T., & Nagai, S. (1998). Use of photosynthetic bacteria for the production of SCP and chemicals from organic wastes. In A.M. Martin (Ed.), *Bioconversion of waste materials to industrial products* (2nd ed.) (pp. 247-292). London: Blackie Academic & Professional.

Ogata, H.Y., Ahmed, K., Garibay, E.S., Chavez, D.R., Furuita, H., & Chong, V.C. (2006). Arachidonic acid distribution in mangrove organisms in the Philippines, Malaysia and Japan. *JIRCAS Working Report No.44*, 45-48.

Ogata, H.Y., Emata, A.C., Garibay, E.S., & Furuita, H. (2004). Notable features of fatty acid composition in mangrove fish species. *JIRCAS Working Report No.35*, 63-74.

Ohta, M., & Watanabe, T. (1996). Dietary energy budgets in carps. *Fisheries Sciences*, 62, 745-753.

Øie, G., Makridis, P., Reitan, K.I., & Olsen, Y. (1997). Protein and carbon utilization of rotifers (*Brachionus plicatilis*) in first feeding of turbot larvae (*Scophthalmus maximus* L.). *Aquaculture*, 153, 103-122.

Øie, G., & Olsen, Y. (1997). Protein and lipid content of the rotifer *Brachionus plicatilis* during variable growth and feeding condition. *Hydrobiologia*, 358, 251-258.

Okamoto, N., Nirotani, H., Sano, T., & Kobayashi, M. (1988). Antiviral activity of extracts of phototrophic bacteria to fish viruses. *Nippon Suisan Gakkaishi*, 54, 2225. Cited in Kobayashi, M., & Kobayashi, M. (1995). Waste remediation and treatment using anoxygenic photosynthetic bacteria. In R.E. Blankenship, M.T. Madigan & C.E. Baucer (Eds.), *Anoxygenic photosynthetic bacteria* (pp. 1269-1282). Dordrecht: Kluwer Academic.

Onal, U., Çelík, İ., & Ergűn, S. (2009). The performance of a small-scale, high-density, continuous system for culturing the rotifer *Brachionus plicatilis*. *Turkish Journal of Veterinary and Animal Sciences*, 34(2), 187-195.

Ozkizilick, S., & Chu, F.E. (1994). Uptake and metabolism of liposomes by *Artemia* nauplii. *Aquaculture*, 128, 131-141.

Panu, T., Anusorn, M., Tawee, W., & Weera, W. (1984). Culture of marble goby (*Oxyeleotris marmoratus*). *DOF Annual Report*, 27-47.

Pfennig, N. (1967). Photosynthetic bacteria. *Annual Review of Microbiology*, 21, 265-324.

Pfennig, N. (1977). Photosynthetic green and purple bacteria: a comparative, systematic survey. *Annual Review of Microbiology*, 31, 275-290.

Phang, S.M. (1990). Algal production from agro-industrial and agricultural waste in Malaysia. *AMBIO*, 19, 415-418.

Pickering, A.D., & Pottinger, T.G. (1989). Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish Physiology and Biochemisrty*, 7, 253-258.

Pisarn, J., & Wichaen, P. (1977). The studies of sand goby (*Oxyeloetris marmoratus* Breeker). In Department of Fisheries (Ed.), *Department of Fisheries, annual report* (pp. 24-44). Bangkok, Thailand: Thai Department of Fisheries.

Planas, M., & Cunha, I. (1999). Larviculture of marine fish: problems and perspectives. *Aquaculture*, 177, 171-190.

Planas, M., Vazquez, J.A., Marquės, J., Pėrez-Lomba, R., Gonzalez, M.P., & Murado, M. (2004). Enhancement of rotifer (*Brachionus plicatilis*) growth by using terrestrial lactic acid bacteria. *Aquaculture*, 240, 313-329.

Polhamus, L.G. (1962). Production of synthetic rubber. In *Rubber*: *botany, production and utilization* (pp. 271-285). London: Leonard Hill (Books) Ltd.

Pomeranz, Y., & Meloan, C.E. (1987). *Food analysis - theory and practice* (2nd ed.). NY: AVI Van Nostrand Reinhold Company Inc.

Prasankok, P., Kanchanakhan, S., & Chutmongkonkul., M. (2002). Characterization of a iridovirus isolated from diseased marble goby *Oxyeleotris marmoratus* (Bleeker, 1985). *Paper presented at the 5th symposium on diseases in Asian aquaculture held from 24-28 November 2002 in Gold Coast, Australia*.

Prasertsan, P., Choorit, W., & Suwanno, S. (1993). Isolation, identification and growth condition of photosynthetic bacteria found in seafood processing wastewater. *World Journal of Microbiology and Biotechnology*, 9, 590-592.

Procarione, L.S., Barry, T.P., & Malison, J.A. (1999). Effect of high rearing density and loading rates on the growth and stress response of juvenile rainbow trout. *North American Journal of Aquaculture*, 61, 91-96.

Puvanendran, V., & Brown, J.A. (2002). Foraging, growth and survival of Atlantic cod, *Gadus morhua*, larvae reared in different light intensities and photoperiods. *Aquaculture*, 214, 131-151.

Rainuzzo, J.R., Reitan, K.I., & Olsen, Y. (1997). The significance of lipids at early stages of marine fish: a review. *Aquaculture*, 155, 103-115.

Rakbankerd (2005). Marble goby; culture and market. Retrieved 1 March 2011, from http://www.rakbankerd.com/agriculture/commerce/new_board01.html?id=6365

Ratledge, C. (2001). Microorganisms as sources of polyunsaturated fatty acids. In F.D. Gunstone (Ed.), *Structured and modified lipids* (pp. 351-399). New York: Dekker.

Ratledge, C., & Wilkinson, S.G. (1989). *Microbial lipids – volume 2*. United Kingdom: Academic Press Limited.

Razlutskij, V.I. (2000). Estimating cladoceran birth rate: use of the egg age distribution to estimate mortality of ovigerous female and eggs. *Hydrobiologia*, 428, 135-144.

Reitan, K.I., Rainuzzo, J.R., Øie, G., & Olsen, Y. (1993). Nutritional effect of algal addition in first-feeding of turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture*, 118, 257-275.

Richmond, A. (2004). *Handbook on microalgal culture: biotechnology and applied phycology*. Iowa: Iowa State Press/Blackwell.

Roberts, T.R. (1989). *The freshwater fishes of Western Borneo (Kalimantan Barat, Indonesia)* (pp. 210). San Francisco: California Academy of Sciences.

Rønnestad, I., Conceição, L.E.C., Aragão, C., & Dinis, M.T. (2003). Free amino acids are absorbed faster and assimilated more efficiently than protein in post larval Senegal sole (*Solea senegalensis*). *Journal of Nutrition*, 130, 2809–2812.

Rønnestad, I., Thorsen, A., & Finn, R.N. (1999). Fish larval nutrition: a review of recent advances in the roles of amino acids. *Aquaculture*, 177, 201–216.

Rotllant, J., Tort, L., Montero, D., Pavlidis, M., Martinez, M., Wendelaar Bongae, S.E., & Balme, P.H.M. (2003). Background colour influence on the stress response in cultured red porgy *Pagrus pagrus*. *Aquaculture*, 223, 129-139.

Ruttner-Kolisko, A. (1972). Rotatorien. *Die Binnengewässer Bd*, 26, 99-234.

Samantaray, K., & Mohantly, S.S. (1997). Interactions of dietary levels of protein and energy on fingerling snakehead, *Channa striata*. *Aquaculture*, 156, 241-249.

Sargent, J., Bell, G., McEvoy, D.T., & Estevez, E. (1999a). Recent developments in the essential fatty acid nutrition of fish. *Aquaculture*, 177, 191-199.

Sargent, J.R., McEvoy, L.A., & Bell, J.G. (1997). Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture*, 155, 85-101.

Sargent, J.R., McEvoy, L.A., Estevez, A., Bell, J.G., Bell, M.V., Henderson, R.J., & Tocher, D.R. (1999b). Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture*, 179, 217-229.

Sargent, J.R., Tocher, R.D., & Bell, J.G. (2002). The lipids. In E.J. Halver & W.R. Hardy (Eds.), *Fish nutrition (3rd ed.)* (pp. 181-246). London, UK: Academic Press.

Sasaki, K., & Nagai, S. (1979). The optimum pH and temperature for the aerobic growth of *Rhodopseudomonas gelatinosa* and vitamin B_{12} and ubiquinone formation on a starch medium. *Journal of Fermentation Technology*, 57(5), 383-386.

Sasaki, K., Noparatnaraporn, N., Hayashi, M., Nishizawa, Y., & Nagai, S. (1981). Single cell protein production by treatment of soybean wastes with *Rhodopseudomonas gelatinosa*. *Journal of Fermentation Technology*, 59, 471-477.

Sasaki, K., Noparatnaraporn, N., & Nagai, S. (1991). Use of photosynthetic bacteria for the production of SCP and chemical from agro-industrial wastes. In A.M. Martin (Ed.), *Bio-conversion of waste materials to industrial products* (pp. 225-263). London: Applied Science.

Sasaki, K., Tanaka, T., & Nagai, S. (1998). Use of photosynthetic bacteria for the production of SCP and chemicals from organic wastes. In A.M. Martin (Ed.), *Bioconversion of waste materials to industrial products* (2nd ed.) (pp. 247-292). London: Blackie Academic & Professional.

Sasikala, C., & Ramana, C.V. (1995). Biotechnological potentials of anoxygenic phototrophic bacteria. I. production of single-cell protein, vitamins, ubiquinones, hormones, and enzymes and use in waste treatment. *Advances in Applied Microbiology*, 41, 173-226.

Sasikala, K., Ramana, C.V., Raghuveer, R., & Kovacs, K.L. (1993). Anoxygenic phototrophic bacteria: physiology and advances in hydrogen production technology. *Advances in Applied Microbiology*, 38, 212-295.

Sawada, H., & Rogers, P.L. (1977). Photosynthetic bacteria in waste treatment-mixed culture studies with *Rhodopseudomonas capsulate*. *Journal of Fermentation Technology*, 55(4), 311-325.

Sayer, D.J.M. (2005). Adaptions of amphibious fish for surviving life out of water. *Fish and Fisheries*, 6, 186-211.

Scott, A.P., & Middleton, C. (1979). Unicellular algae as a food for turbot (*Scophthalmus maximus* L.) larvae - the importance of dietary long chain polyunsaturated fatty acids. *Aquaculture*, 18, 227-240.

Scragg, A. (1999). *Environmental biotechnology* (pp. 6). Singapore: Pearson Education Limited.

Senoo, S. (2003). Artificial seed production of marble goby, *Oxyeleotris marmoratus* - II. Fish culture in Southeast Asia 30. *Aquatic Network*, 9, 66-71.

Senoo, S., Ang, K.J., & Cheah, S.H. (1993a). Natural spawning of marble goby, *Oxyeleotris marmoratus* under artificial conditions. *Malaysian Fisheries Society Fishmail*, 5, 16-18.

Senoo, S., Ang, K.J., & Cheah, S.H. (1993b). Deformation of marble goby *Oxyeleotris marmoratus* seed reared under tank condition. In S.B. Japar, F.M. Yusoff, M.S. Mohd Zaki & T.Petri (Eds.), *Fisheries and the environment: beyond 2000* (pp. 229-233). Serdang, Malaysia: Universiti Putra Malaysia.

Senoo, S., Ang, K.J., & Cheah, S.H. (1997). Egg collection from small-sized marble goby, *Oxyeleotris marmoratus* by natural spawning method in tanks. In B.F.M. Japar Sidik, T.P. Yussoff & M.S. Mohd Zaki (Eds.) (pp. 251-256). *Fisheries and the environment: beyond 2000*. Serdang, Malaysia: Universiti Putra Malaysia.

Senoo, S., Ang, K.J., & Kaneko, M. (1994a). Artificial seed production technique of marble goby *Oxyeleotris marmoratus*: Aquaculture practices in Malaysia. *Malaysian Fisheries Society* (pp. 1-15).

Senoo, S., Ang, K.J., & Kawamura, G. (1994b). Development of sense organs and mouth and feeding of reared marble goby *Oxyeleotris marmoratus* larvae. *Fisheries Science*, 60(4), 361-368.

Senoo, S., Cheah, S.H., & Ang, K.J (1992). Spawning the ketutu. *Malaysian Society Fishmail*, 4, 3-5.

Senoo, S., Kaneko, M., Cheah, S.H., & Ang, K.J. (1994c). Egg development, hatching and larval development of marble goby *Oxyeleotris marmoratus* under artificial rearing conditions. *Fisheries Science*, 60(1), 1-8.

Senoo, S., Sow, S.H., & Mukai, Y. (2008). Effects of different salinity levels on the survival and growth of marble goby, *Oxyeleotris marmoratus* larvae. *Aquaculture Science*, 56(3), 423-432.

Sergiusz, C., Sagiv, K., Konrad, D., & David, C. (1999). Growth, survival, and quality of juvenile walleye *Stizostedion vitreum* as influenced by n-3 HUFA enriched *Artemia* nauplii. *Aquaculture*, 178, 1-2.

Shaliza, I., Vikineswary, S., Azad, S.A., & Chong, L.L. (2006). The effects of light intensity, inoculum size, and cell immobilization on the treatment of sago effluent with *Rhodopseudomonas palustris* strain B1. *Biotechnology and Bioprocess Engineering*, 11, 377-381.

Shi, C.W. (2009). Biohydrogen production from palm oil mill effluent as substrate by *Rhodopseudomonas palustris* PBUM 001. Unpublished master's thesis, Faculty of Engineering, University of Malaya, Kuala Lumpur.

Shields, R.J. (2001). Larviculture of marine finfish in Europe. *Aquaculture*, 200(1-2), 55-88.

Shipman, R.H., Fan, L.T., & Kao, I.C. (1977). Single cell protein production by photosynthetic bacteria. *Advance Applied Microbiology*, 21, 161-183.

Shipman, R.H., Kao, I.C., & Fan, L.T. (1975). Single cell protein production by photosynthetic bacteria cultivation in agricultural by products. *Biotechnology and Bioengineering*, 17(11), 1561-1570.

Simon, C.M. (1978). The culture of the diatom *Chaetoceros gracilis* and its use as a food for penaeid protozoeal larvae. *Aquaculture*, 14, 105-113.

Singh, A., & Ward, O.P. (1997). Microbial production of docosahexaenoic acids (DHA, C22:6). *Advances in Applied Microbiology*, 45, 271-312.

Smith, P.F. (1988). Archaebacteria and other specialized bacteria. In C. Ratledge & S.G. Wilkinson (Eds.), *Microbial lipids – volume 1*. Great Britain: Academic Press Limited.

Snell, T.W. (1991). Improving the design of mass culture systems for the rotifer *Brachionus plicatilis*. In *Rotifer and Microalgae Culture Systems* (pp. 61-71). Honolulu, HI, The Oceanic Institute: Proceedings of a U.S. - Asia Workshop.

Snell, T.W., & Boyer, E.M. (1988). Thresholds for mictic female production in the rotifer *Brachionus plicatilis* (Muller). *Journal of Experimental Marine Biology and Ecology*, 124, 73-85.

Sompong, H. (1980). Life history of sand goby (*Oxyeleotris marmoratus* Blekker) (in Thai). *Technical Paper, Royal Thai Department of Fisheries* no. 13, pp. 42.

Sorgeloos, P., & Lavens, P. (1996). Manual on the production and use of live food for aquaculture. *Fisheries technical paper*, volume 361 (pp. 9-100). Rome: Food and Agriculture Organization of the United Nation.

Southgate, P.C., & Lou, D.C. (1995). Improving the n-3 HUFA composition of *Artemia* using microcapsules containing marine oil. *Aquaculture*, 134, 91-99.

Srivastava, A., Hamre, K., Stoss, J., Chakrabarti, R., & Tonheim, S.K. (2005). Protein content and amino acid composition of the live feed rotifer (*Brachionus plicatilis*): with emphasis on the water soluble fraction. *Aquaculture*, 254(1-4), 534-543.

Stainer, R.Y., Pfennig, N., & Truper, H.G. (1981). Introduction to the phototrophic prokaryotes. In M.P. Starr, H. Stolp, H.G. Truper, A. Balows & H.G. Schlegel (Eds.), *The prokaryotes* (pp. 197-211). New York: Springer-Verlag.

Standard methods for analysis of oils, fats and derivates, International Union of Pure and Applied Chemistry, IUPAC method 2.301 (7th ed.) (1987). Blackwell Scientific Publications.

Starkweather, P.L., Gilbert, J.J., & Frost, T.M. (1979). Bacterial feeding in the rotifer *Brachionus calyciflorus*: clearance and ingestion rates, behavior and population dynamics. *Oecologia*, 44, 26-30.

Støttrup, J.G., & Attramadal, Y. (1992). The influence of different rotifer and *Artemia* enrichment diets on growth, survival and pigmentation in turbot (*Scophthalmus maximus* L.) larvae. *Journal of the World Aquaculture Society*, 23, 307-316.

Suantika, G., Dhert, P., De Wolf, T., & Sorgeloos, P. (2000a). The use of a recirculation system for rotifer production on a commercial scale. In R. Flos & L. Creswell (Eds.), *Responsible aquaculture in the new millenium, AQUA 2000* (pp. 685). Nice, France: European Aquaculture Society, Special Publication 28.

Suantika, G., Dhert, P., Nurhudah, M., & Sorgeloos, P. (2000b). High-density production of the rotifer *Brachionus plicatilis* in a recirculating system: consideration of water quality, zootechnical and nutritional aspects. *Aquacultural Engineering*, 21, 201-214.

Sugden, L.G. (1973). Feeding ecology of Pintail Gadwall, American Wigeon and Lesser Scaup ducklings. *Canadian Wildlife Services Report*, 24, 45.

Sugiyama, S., Staples, D., & Funge-Smith, S.J. (2004). *Status and potential of fisheries and aquaculture in Asia and the Pacific* (pp. 53). FAO regional office for Asia and the Pacific: RAP Publication, Bangkok.

Supamataya, M. (1984). The study of disease in marble goby (*Oxyeleotris marmoratus* Bleerker) in cage culture and some environmental factors related to infection. Unpublished master's thesis, Faculty of Fisheries Sciences, Kasetsart University.

Suwansart, P. (1979). Cage culture of sand goby, *Oxyeleotris marmoratus* (Blk) at Nan River. *Thailand Fisheries Gazatte*, 29, 51-56.

Suzuki, H., Daimon, M., Awano, T., Umekage, S., Tanaka, T., & Kikuchi, Y. (2009). Characterization of extracellular DNA production and flocculation of the marine photosynthetic bacterium *Rhodovulum sulfidophilum*. *Applied Microbiology and Biotechnology*, 84, 349-356.

Svensson, O., Magnhagen, C., Forsgren, E., & Kvarnemo, C. (1998). Parental behaviour in relation to the occurrence of sneaking in the common goby. *Animal Behaviour*, 56, 175-179.

Ta, Y.W., Abdul Wahab, M., Jamaliah, M.J., & Nurina, A. (2009). A holistic approach to managing palm oil mill effluent (POME): Biotechnological advances in the sustainable reuse of POME. *Biotechnology Advances*, 27, 40-52.

Takita, T. (1975). Spawning and embryonic development of Synechogobuis hasta in an aquarium with description of larvae and juvenile. *Japanese Journal of Ichthyology*, 22, 31-39.

Tan, O.K., & Lam, T.J. (1973). Induced breeding and early development of the marble goby (*Oxyeleotris marmoratus*, BLK). *Aquaculture*, 2, 411-423.

Taufik, I., Azwar, Z.I., & Ikhsan, K. (2002). Effect of different colour of tank on growth and survival in *Oxyeleotris marmoratus*, Blkr. *Research report*. Bogor, Indonesia: Centre of Freshwater Research.

Tavarutmaneegul, P., & Lin, C.K. (1988). Breeding and rearing of sand goby (*Oxyeleotris marmoratus*, Bleeker). *Aquaculture*, 60, 299-305.

Ter Braak, C.J.F., & Smilauer, P. (2002). *CANOCO Reference manual and CanoDraw for windows user's guide: software for canonical community ordination (version 4.5)* (pp 500). Ithaca, New York, USA: Microcomputer Power.

Theilacker, G.H., & Kimball, A.S. (1984). Comparative quality of rotifers and copepods as foods for larval fishes. *CalCOFI Reports*, 25, 80-86.

Tort, L., Sunyer, J.O., Gómez, E., & Molinero, A. (1996). Crowding stress induced changes in serum haemolytic and agglutinating activity in the gilthead sea bream *Sparus aurata*. *Veterinary Immunology and Immunopathology*, 51, 179-188.

Van, M.V., Abol-Munafi, A.B., Effendy, A.W.M., & Awang, S.M. (2005). The effect of different diets on proteolytic enzymes activity of early marble goby (*Oxyeleotris marmoratus*) larvae. *Journal of Animal Veterinary Advance*, 4(10), 835-838.

Van Niel, C.B. (1957). Bergey's manual of determinative bacteriology. In H. Kojima & Y.K. Lee (Eds.), *Photosynthetic microorganisms in environmental biotechnology* (pp. 12). Hong Kong: Springer-Verlag, Hong Kong Ltd.

Van Stappen, G. (1996). Introduction, biology and ecology of *Artemia*. In P. Lavens & P. Sorgeloos (Eds.), M*anual on the production and use of live food for aquaculture* (pp.79-106). ROME: FAO, Fisheries Technical Paper No. 361.

Vijayaraghavan, K., & Ahmad, D. (2006). Biohydrogen generation from palm oil mill effluent using anaerobic contact filter. *International Journal of Hydrogen Energy*, 31(10), 1284-1291.

Wahyuningrum, R.D. (1991). Development of sand goby (*Oxyeleotris marmoratus* BLKR). Unpublished BSc.'s thesis, University of Bogor Agriculture.

Waspada, Mayunar, & Fatoni, T. (1991). Nutrient enrichment of rotifers, *Brachionus plicatilis*, to support fry production of grouper, *Epinephelus fuscoguttatus*. *Journal Penelitian Budidaya Pantai*, 7, 73-79. (In Indonesian, English abstract).

Watanabe, M., Sasaki, K., Nakashimada, Y., Kakizono, T., Noparatnaraporn, N., & Nishio, N. (1998). Growth and flocculation of a marine photosynthesis bacterium *Rhodovulum* sp. *Applied Microbiology and Biotechnology*, 50, 682-691.

Watanabe, T., Kitajima, C., & Fujita, S. (1983a). Nutritional values of live organisms used in Japan for mass propagation of fishes: a review. *Aquaculture*, 34, 115-143.

Watanabe, T., Oowa, F., Kitajima, C., & Fujita, S. (1978). Nutritional quality of brine shrimp *Artemia salina*, as a living feed from the viewpoint of essential fatty acids for fish. *Bulletin of the Japanese Society for the Science of Fish*, 44, 1115-1121.

Watanabe, T., Tamiya, T., Oka, A., Hirata, M., Kitajima, C., & Fujita, S. (1983b). Improvement of the dietary value of live foods for fish larvae by feeding them on ω3 highly unsaturated fatty acids and fat soluble vitamins. *Bulletin of the Japanese Society for the Science of Fish*, 49, 471-479.

Wendy, F., & Kevan, L.M. (1991). Rotifer and microalgae culture system. Proceedings of a U.S. - Asia workshop.

Widanarni. (1990). Early development of sand goby, *Oxyeleotris marmoratus* (Blkr) larvae. Unpublished master's thesis, University of Bogor Agriculture.

Widdel, F., Schnell, S., Heising, S., Ehrenreich, A., Assmus, B., & Schink, B. (1993). Ferrous iron oxidation by anoxygenic phototrophic bacteria. *Nature*, 362, 834-836.

Widiyati, A., & Djajasewaka, H. (Eds.). (1992). *Proceeding of Freshwater Aquaculture Research: The effect of artificial food on the growth and survival rate of sand goby (Oxyeleotris marmoratus, Blkr) reared in conducted water temperature*. Bogor, Indonesia.

Wilson, R.P. (1995). Lipid nutrition of finfish. In C. Lim & D. J. Sessa (Eds.), *Nutrition and utilization technology in aquaculture* (pp. 74-81). Champaign: AOCS Press.

Wolfinbarger, W.C. (1999). Influences of biotic and abiotic factors on seasonal succession of zooplankton in Hugo Reservoir, Oklahoma, USA. *Hydrobiologia*, 400, 13-31.

Wong, Y.S., Kadir, M.O.A.B., & Teng, T.T. (2009). Biological kinetics evaluation of anaerobic stabilization pond treatment of palm oil mill effluent. *Bioresource Technology*, 100, 4969-4975.

Wu, T.Y., Mohammad, A.W., Md Jahim, J., & Annuar, N. (2007). Palm oil mill effluent (POME) treatment and bioresources recovery using ultrafiltration membrane: effect of pressure on membrane fouling. *Biochemical Engineering Journal*, 35, 309-317.

Yoshimura, K., Kitajima, C., Miyamoto, Y., & Kishimoto, G. (1994). Factors inhibiting growth of the rotifer *Brachionus plicatilis* in high density cultivation by feeding condensed *Chlorella*. *Nippon Suisan Gakhaishi*, 60, 207-213.

Yoshimura, K., Ohmori, Y., Yoshimatsu, T., Tanaka, K., & Ishizaki, A. (1996). On the aeration method in high density culture of rotifer *Brachionus rotundiformis*. *Nippon Suisan Gakkaishi*, 62(6), 897-903.

Yoshimura, K., Tanaka, K., & Yoshimatsu, T. (2003). A novel culture system for the ultra-high-density production of the rotifer, *Brachionus rotundiformis* - a preliminary report. *Aquaculture*, 227, 165-172.

Yu, J.P., Hino, A., Hirano, R., & Hirayama, K. (1988). Vitamin B_{12}-producing bacteria as a nutritive complement for a culture of the rotifer *Brachionus plicatilis*. *Nippon Suisan Gakkaishi*, 54, 1873-1880.

Yu, J.P., & Hirayama, K. (1986). The effect of un-ionized ammonia on the population growth of the rotifer in mass culture. *Nippon Suisan Gakkaishi*, 52, 1509-1513.

Yukov, V.V., & Beatty, T. (1998). Aerobic anoxygenic phototrophic bacteria. *Microbiology and Molecular Biology Reviews*, 62, 695-724.

Zadariana, J. (2009). Simultaneous treatment of palm oil mill effluent and biohydrogen production using *Rhodopseudomonas palustris* PBUM001. Unpublished master's thesis, Faculty of Engineering, University of Malaya, Kuala Lumpur.

Zadariana, J., Mohamad Suffian, M.A., Shaliza, I., & Vikineswary, S. (2009). Optimization of phototrophic hydrogen production *Rhodopseudomonas palustris* PBUM001 via statistical experimental design. *International Journal of Hydrogen Energy*, 34(7), 7502-7512.

Zar, J.H. (1999). *Biostatistical analysis* (4th ed.). Englewood Cliffs, NJ, USA: Prentice Hall.

APPENDICES

Chapter 3: Selection of suitable phototrophic bacterium for aquaculture

Appendix I - 112 medium (Gest & Favinger, 1983)

K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Yeast extract	10.0 g
NaCl	30.0 g
Agar	20.0 g
Distilled water	1 L

pH was adjusted to 7.0 - 7.2 prior to autoclaving.

Appendix II - Proximate composition, fatty acid and amino acid profiles of raw POME

a) Proximate composition of raw POME (g/100 g dry sample of raw POME)

Proximate composition	Raw (POME) (%)[a]	Raw POME (%)[b]
Protein	12.75 ± 1.30	9.07 ± 0.15
Lipid	10.21 ± 1.24	13.21 ± 0.12
Carbohydrate	29.55 ± 2.44	32.12 ± 0.12
Ash	14.88 ± 1.35	20.55 ± 0.14
Moisture	6.99 ± 0.14	6.75 ± 0.03
Nitrogen free extract	26.39 ± 2.33	19.47 ± 0.10
Total carotene	0.019 ± 0.001	NA

b) Fatty acid composition of raw POME (g/100 g lipid)

Structure	FAME	Raw POME (%)[a]	Raw POME (%)[b]
Saturated fatty acids			
C 8:0	Caprylic	2.37 ± 0.06	NA
C 10:0	Capric	4.29 ± 0.09	4.29 ± 0.03
C 12:0	Lauric	3.22 ± 0.06	9.22 ± 0.03
C 14:0	Myristic	12.66 ± 0.12	12.66 ± 0.11
C 15:0	Pentadecanoic	2.21 ± 0.04	NA
C 16:0	Palmitic	22.45 ± 1.88	14.45 ± 0.12
C 17:0	Heptadecanoic	1.39 ± 0.03	1.39 ± 0.02
C 18:0	Stearic	10.41 ± 0.54	11.41 ± 0.08
C 20:0	Arachidic	3.56 ± 0.06	7.56 ± 0.03
C 22:0	Behenic	NA	2.62 ± 0.03
Total		62.56	63.6
Unsaturated fatty acids			
C 17:1	Cis-10-Heptadecanoic	1.12 ± 0.03	1.12 ± 0.02
C 18:1n9c	Oleic	14.54 ± 1.25	8.54 ± 0.06
Total		15.66	9.66
Polyunsaturated fatty acids			
C 18:2n6c	Linoleic (Cis)	9.53 ± 1.20	9.53 ±0.05
C 18:3n3	α-Linolenic	4.72 ± 0.54	4.72 ± 0.04
C 18:3n6	β-Linolenic	0	NA
C 20:3n6	Cis-8,11,14-Eicosatrienoic	2.04 ± 0.08	1.49 ± 0.02
C 20:4n6	Arachidonic (ARA)	1.12 ± 0.03	1.12 ± 0.03
C 20:5n3	Cis-5,8,11,14,17-eicosapentaenoic (EPA)	0.36 ± 0.06	0.36 ± 0.02
Total		17.77	17.22

[a] Habib et al., 1997; [b] Habib et al., 1998; NA - Not Available

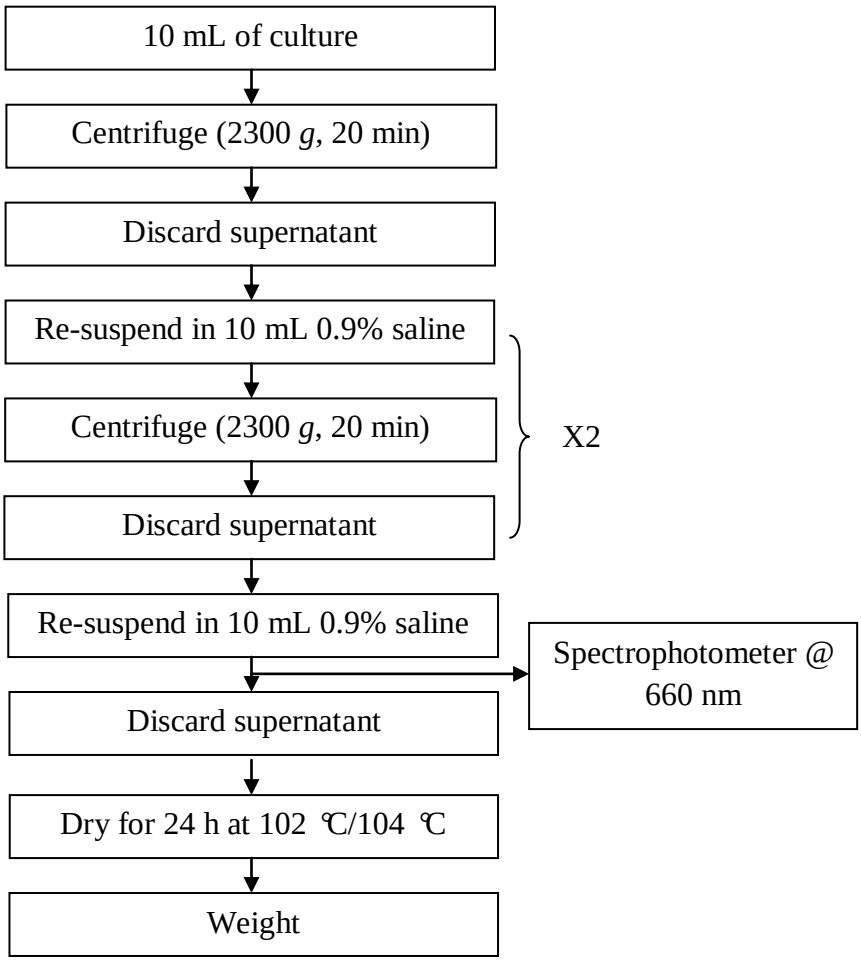
c) Amino acid composition of raw POME (%)

Amino acid profile	Raw POME (%)[a]	Raw POME (%)[b]
Essential amino acids		
Histidine	1.43 ± 0.14	1.43 ± 0.04
Threonine	2.58 ± 0.25	2.58 ± 0.05
Valine	3.56 ± 0.24	3.56 ± 0.06
Methionine	6.88 ± 0.55	6.88 ± 0.15
Lysine	2.66 ± 0.14	2.66 ± 0.14
Isoleucine	4.53 ± 0.33	4.53 ± 0.11
Leucine	4.86 ± 0.22	6.86 ± 0.15
Phenylalanine	3.20 ± 0.15	3.20 ± 0.07
Arginine	4.25 ± 0.42	4.15 ± 0.10
Tryptophan	1.26 ± 0.04	1.26 ± 0.05
Total	35.21	37.11
Non-essential amino acids		
Aspartic acid	9.66 ± 0.15	9.66 ± 0.19
Serine	6.86 ± 0.14	6.86 ± 0.15
Glutamic acid	10.88 + 0.13	10.88 ± 0.21
Glycine	9.43 ± 0.18	9.43 ± 0.17
Alanine	7.70 ± 0.04	7.70 ± 0.16
Proline	4.57 ± 0.11	4.57 ± 0.10
Tyrosine	3.16 ± 0.19	3.26 ± 0.06
Cystine	3.37 ± 0.07	3.37 ± 0.06
Total	55.63	55.73

[a] Habib et al., 1997; [b] Habib et al., 1998

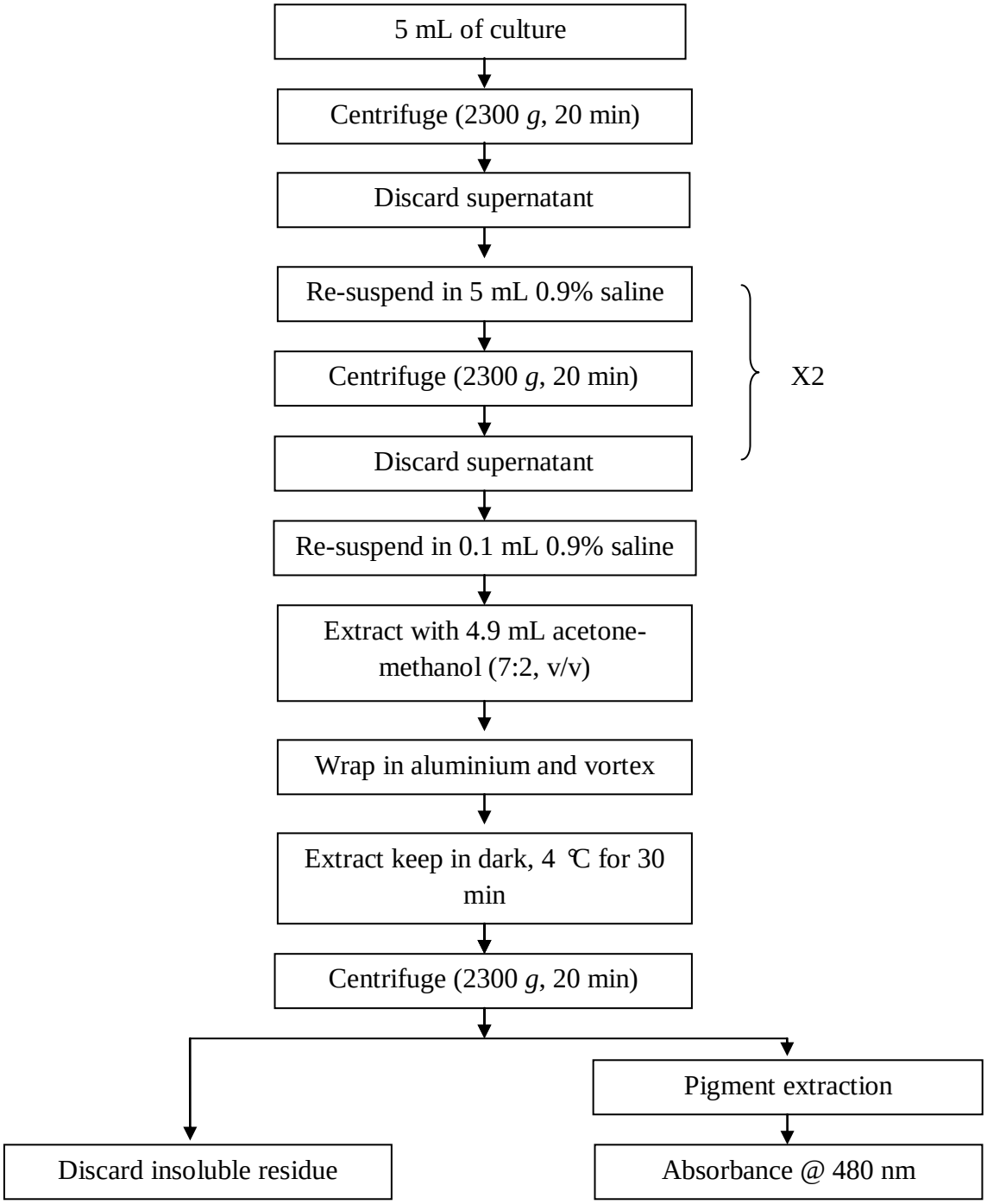
Appendix III – Dry weight (DW) of bacterial cell biomass (Sawada & Rogers, 1977)

The cell DW was determined by taking 10 mL of homogenous mixed culture sample into a previously weighed centrifuged tube and centrifuged at 2300 *g* for 20 min. The cell was re-suspended and re-centrifuged twice at same speed. The packed cell mass in the tube was oven-dried at 102 ℃ overnight. The tube was re-weighed after cooling in a desiccator. The measurements were done in triplicates. The method is as follows:



Appendix IV - Carotenoids (Jensen & Jensen, 1971)

The total bacterial carotenoids concentration was determined by the extraction procedure. Five millilitres of culture sample was centrifuged and re-suspended twice to measure its carotenoids at OD of 480 nm. The method is as follows:



Formula for total amount of carotenoids:

C = D.v.f. (10/2500) / DW of sample (g)

Where,

C = Total carotenoids (mg/g)

D = OD (SD) at 480 nm

f = dilution factor of sample (only if OD > 0.8)

v = Total volume (mL)

2500 = Average extraction coefficient for carotenoids

Appendix V - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effect of media on the production of bacterial biomass and carotenoids

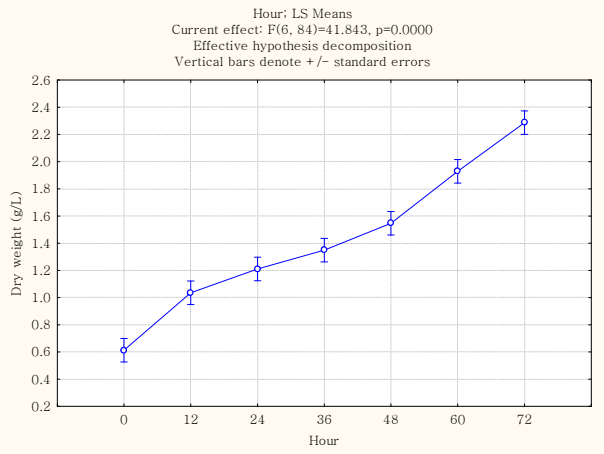
a) Dry biomass

(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	Univariate Tests of Significance for Dry weight (g/L) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	255.7448	1	255.7448	1902.413	0.000000
Time	33.7499	6	5.6250	41.843	0.000000
Culture medium	3.7642	5	0.7528	5.600	0.000168
Time*Culture medium	19.9110	30	0.6637	4.937	0.000000
Error	11.2923	84	0.1344		

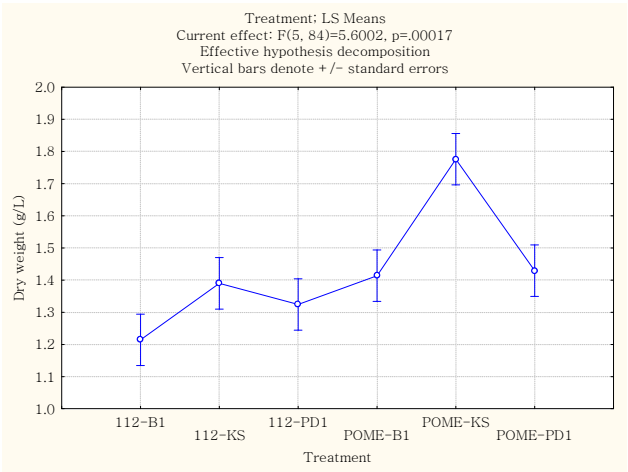
(ii) Time

Cell No.	Tukey HSD test; variable Dry weight (g/L) Homogenous Groups, alpha = .05000 Error: Between MS = .13443, df = 84.000					
	Hour	Dry weight (g/L) Mean	1	2	3	4
1	0	0.613333				****
2	12	1.035556	****			
3	24	1.210556	****	****		
4	36	1.349444	****	****		
5	48	1.547222		****		
6	60	1.929444			****	
7	72	2.287222			****	



(iii) Culture medium

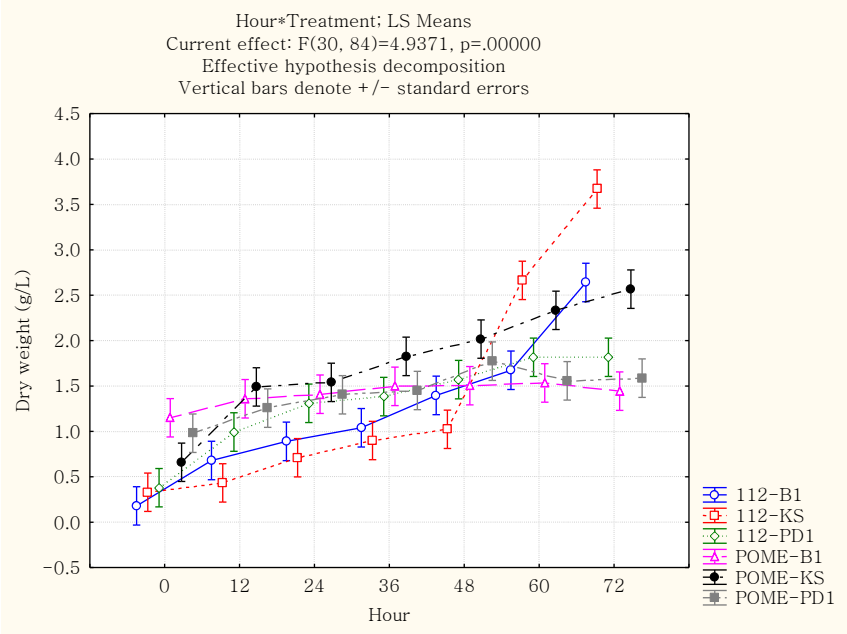
Cell No.	Tukey HSD test; variable Dry weight (g/l Homogenous Groups, alpha = .05000 Error: Between MS = .13443, df = 84.0(			
	Treatment	Dry weight (g/L) Mean	1	2
1	112-B1	1.214286	****	
3	112-PD1	1.324286	****	
2	112-KS	1.390000	****	
4	POME-B1	1.413810	****	
6	POME-PD1	1.429524	****	
5	POME-KS	1.776190		****



(iv) Time x Culture medium interaction

Tukey HSD test; variable Dry weight (g/L)
Homogenous Groups, alpha = .05000 (Non-Exhaustive Search)
Error: Between MS = .13443, df = 84.000

Cell No.	Hour	Treatment	Dry weight (g/L) Mean	1	2	3	4	5	6	7	8	9	10
1	0	112-B1	0.180000	****									
2	0	112-KS	0.330000	****	****								
3	0	112-PD1	0.380000	****	****	****							
8	12	112-KS	0.433333	****	****	****							
5	0	POME-KS	0.660000	****	****	****	****						
7	12	112-B1	0.680000	****	****	****	****						
14	24	112-KS	0.710000	****	****	****	****						
13	24	112-B1	0.890000	****	****	****	****	****					
20	36	112-KS	0.900000	****	****	****	****	****					
6	0	POME-PD1	0.980000	****	****	****	****	****					
9	12	112-PD1	0.993333	****	****	****	****	****					
26	48	112-KS	1.023333	****	****	****	****	****					
19	36	112-B1	1.040000	****	****	****	****	****					
4	0	POME-B1	1.150000	****	****	****	****	****	****				
12	12	POME-PD1	1.256667	****	****	****	****	****	****				
15	24	112-PD1	1.310000	****	****	****	****	****	****				
10	12	POME-B1	1.360000	****	****	****	****	****	****	****			
21	36	112-PD1	1.383333	****	****	****	****	****	****	****			
25	48	112-B1	1.396667		****	****	****	****	****	****			
18	24	POME-PD1	1.403333		****	****	****	****	****	****			
16	24	POME-B1	1.410000		****	****	****	****	****	****			
40	72	POME-B1	1.443333		****	****	****	****	****	****	****		
24	36	POME-PD1	1.450000		****	****	****	****	****	****	****	****	
11	12	POME-KS	1.490000		****	****	****	****	****	****	****	****	
22	36	POME-B1	1.496667		****	****	****	****	****	****	****	****	
28	48	POME-B1	1.503333		****	****	****	****	****	****	****	****	
34	60	POME-B1	1.533333		****	****	****	****	****	****	****	****	
17	24	POME-KS	1.540000		****	****	****	****	****	****	****	****	
36	60	POME-PD1	1.556667			****	****	****	****	****	****	****	
27	48	112-PD1	1.570000			****	****	****	****	****	****	****	
42	72	POME-PD1	1.586667			****	****	****	****	****	****	****	
31	60	112-B1	1.673333				****	****	****	****	****	****	
30	48	POME-PD1	1.773333				****	****	****	****	****	****	
33	60	112-PD1	1.816667				****	****	****	****	****	****	
39	72	112-PD1	1.816667				****	****	****	****	****	****	
23	36	POME-KS	1.826667				****	****	****	****	****	****	
29	48	POME-KS	2.016667					****	****	****	****	****	
35	60	POME-KS	2.333333						****	****	****	****	
41	72	POME-KS	2.566667							****	****	****	****
37	72	112-B1	2.640000								****	****	****
32	60	112-KS	2.663333									****	****
38	72	112-KS	3.670000										****



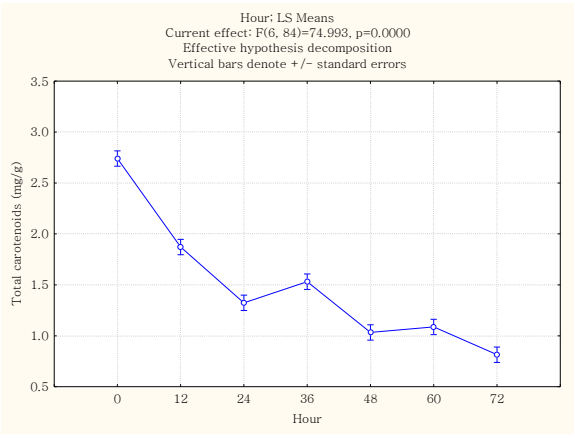
b) Total carotenoids

(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	Univariate Tests of Significance for Total carotenoids (mg/g) Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	278.2808	1	278.2808	2711.851	0.00
Time	46.1734	6	7.6956	74.993	0.00
Culture medium	42.2676	5	8.4535	82.380	0.00
Time*Culture medium	39.7015	30	1.3234	12.896	0.00
Error	8.6198	84	0.1026		

(ii) Time

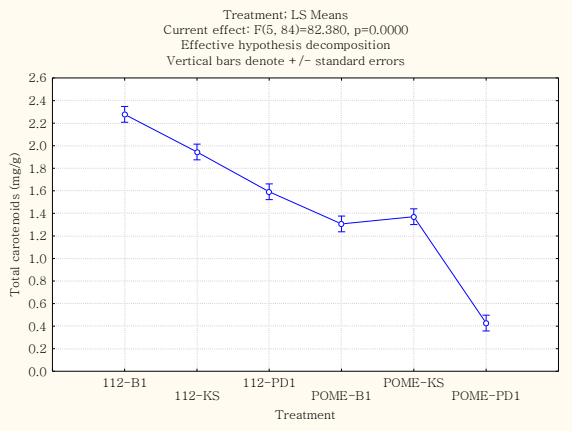
Cell No.	Tukey HSD test; variable Total carotenoids (mg/g) Homogenous Groups, alpha = .05000 Error: Between MS = .10262, df = 84.000 Hour	Total carotenoids (mg/g) Mean	1	2	3	4	5
7	72	0.814688	****				
5	48	1.033425	****	****			
6	60	1.087350	****	****			
3	24	1.323615		****	****		
4	36	1.531566			****		
2	12	1.871892				****	
1	0	2.740362					****



(iii) Culture medium

Tukey HSD test; variable Total carotenoids (mg/g)
Homogenous Groups, alpha = .05000
Error: Between MS = .10262, df = 84.000

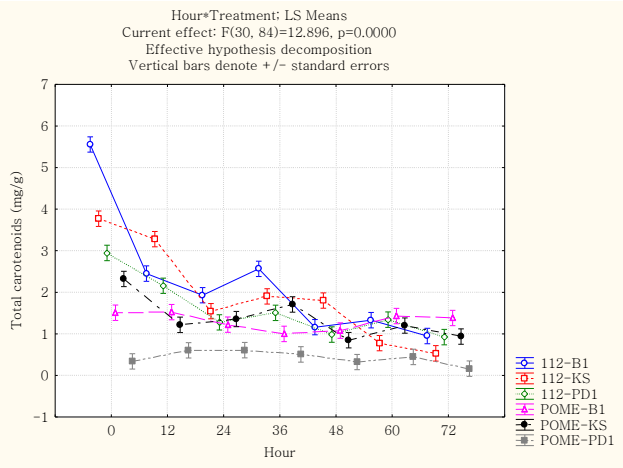
Cell No.	Treatment	Total carotenoids (mg/g) Mean	1	2	3	4
6	POME-PD1	0.427648		****		
4	POME-B1	1.306596	****			
5	POME-KS	1.369920	****			
3	112-PD1	1.591416	****			
2	112-KS	1.943829			****	
1	112-B1	2.277362				****



(iv) Time x Culture medium interaction

Tukey HSD test; variable Total carotenoids (mg/g)
Homogenous Groups, alpha = .05000 (Non-Exhaustive Search)
Error: Between MS = .10262, df = 84.000

Cell No.	Hour	Treatment	Total carotenoids (mg/g) Mean	1	2	3	4	5	6	7	8	9	10	1
42	72	POME-PD1	0.164316	****										
30	48	POME-PD1	0.322954	****	****									
6	0	POME-PD1	0.338776	****	****									
36	60	POME-PD1	0.445452	****	****	****								
24	36	POME-PD1	0.507594	****	****	****	****							
38	72	112-KS	0.533070	****	****	****	****							
12	12	POME-PD1	0.605046	****	****	****	****							
18	24	POME-PD1	0.609401	****	****	****	****							
32	60	112-KS	0.776700	****	****	****	****	****						
29	48	POME-KS	0.850153	****	****	****	****	****	****					
39	72	112-PD1	0.923770	****	****	****	****	****	****	****				
41	72	POME-KS	0.935686	****	****	****	****	****	****	****				
37	72	112-B1	0.949357	****	****	****	****	****	****	****				
27	48	112-PD1	0.984377	****	****	****	****	****	****	****				
22	36	POME-B1	1.000397	****	****	****	****	****	****	****				
28	48	POME-B1	1.079774	****	****	****	****	****	****	****				
25	48	112-B1	1.161689	****	****	****	****	****	****	****	****			
35	60	POME-KS	1.200070	****	****	****	****	****	****	****	****			
11	12	POME-KS	1.217460	****	****	****	****	****	****	****	****			
16	24	POME-B1	1.222067	****	****	****	****	****	****	****	****			
15	24	112-PD1	1.277810		****	****	****	****	****	****	****	****		
31	60	112-B1	1.329798		****	****	****	****	****	****	****	****		
33	60	112-PD1	1.342596		****	****	****	****	****	****	****	****		
17	24	POME-KS	1.357022		****	****	****	****	****	****	****	****		
40	72	POME-B1	1.381928		****	****	****	****	****	****	****	****		
34	60	POME-B1	1.429485			****	****	****	****	****	****	****	****	
21	36	112-PD1	1.506125			****	****	****	****	****	****	****	****	*
4	0	POME-B1	1.509565				****	****	****	****	****	****	****	*
10	12	POME-B1	1.522956				****	****	****	****	****	****	****	*
14	24	112-KS	1.545389				****	****	****	****	****	****	****	*
23	36	POME-KS	1.707834					****	****	****	****	****	****	*
26	48	112-KS	1.801606					****	****	****	****	****	****	*
20	36	112-KS	1.902315						****	****	****	****	****	*
13	24	112-B1	1.930000							****	****	****	****	*
9	12	112-PD1	2.157864								****	****	****	*



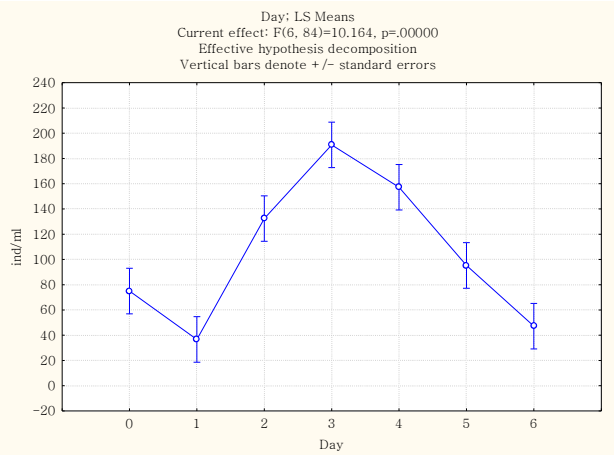
Appendix VI - Summary results of two-way ANOVA and posthoc Tukey HSD test on the evaluation of biomass of PB grown in 112 medium or POME as live microbial feed for rotifer culture

(i) Main effects (shaded area indicates significance at P < 0.01)

Univariate Tests of Significance for ind/mL Sigma-restricted parameterization Effective hypothesis decomposition					
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1387261	1	1387261	236.8519	0.000000
Day of culture	357188	6	59531	10.1640	0.000000
Feed	223910	5	44782	7.6458	0.000006
Day of culture*Feed	411220	30	13707	2.3403	0.001258
Error	491995	84	5857		

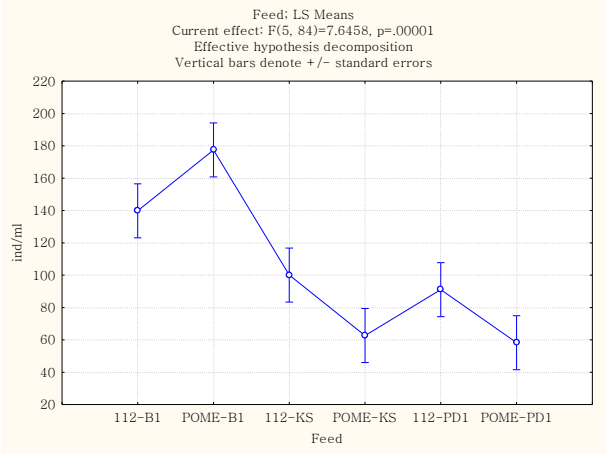
(ii) Day of culture

Tukey HSD test; variable ind/ml Homogenous Groups, alpha = .05000 Error: Between MS = 5857.1, df = 84.00						
Cell No.	Day	ind/ml Mean	1	2	3	4
2	1	36.6667	****			
7	6	47.1667	****			
1	0	75.0000	****	****		
6	5	95.2778	****	****	****	
3	2	132.3889		****	****	****
5	4	157.2222			****	****
4	3	190.7778				****



(iii) Feed

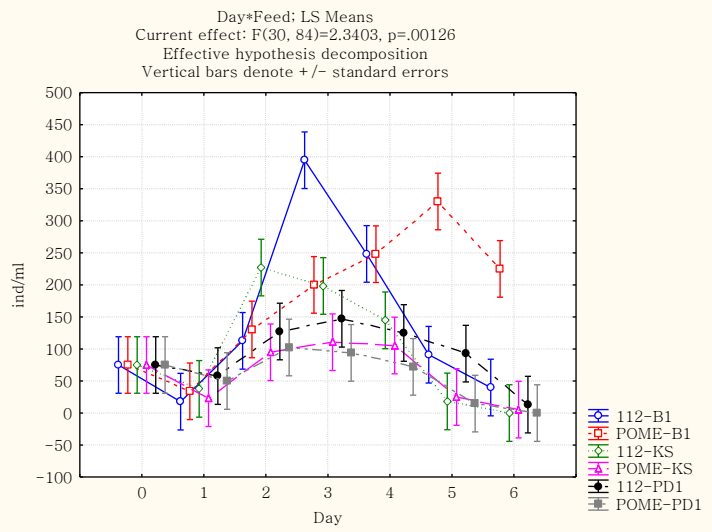
Tukey HSD test; variable ind/ml Homogenous Groups, alpha = .05000 Error: Between MS = 5857.1, df = 84.00					
Cell No.	Feed	ind/ml Mean	1	2	3
6	POME-PD1	58.2857	****		
4	POME-KS	62.7143	****		
5	112-PD1	91.0952	****	****	
3	112-KS	100.0952	****	****	
1	112-B1	139.8571		****	****
2	POME-B1	177.5238			****



(iv) Day of culture x Feed interaction

Tukey HSD test; variable ind/ml
Homogenous Groups, alpha = .05000 (Non-Exhaustive Sear
Error: Between MS = 5857.1, df = 84.000

Cell No.	Day	Feed	ind/ml Mean	1	2	3
39	6	112-KS	0.0000	****		
42	6	POME-PD1	0.0000	****		
40	6	POME-KS	5.3333	****		
41	6	112-PD1	13.0000	****		
36	5	POME-PD1	14.6667	****		
7	1	112-B1	17.6667	****		
33	5	112-KS	18.0000	****		
10	1	POME-KS	23.0000	****		
34	5	POME-KS	25.0000	****		
8	1	POME-B1	34.0000	****		
9	1	112-KS	37.6667	****		
37	6	112-B1	39.6667	****		
12	1	POME-PD1	50.0000	****		
11	1	112-PD1	57.6667	****		
30	4	POME-PD1	72.0000	****		
4	0	POME-KS	75.0000	****		
3	0	112-KS	75.0000	****		
2	0	POME-B1	75.0000	****		
5	0	112-PD1	75.0000	****		
6	0	POME-PD1	75.0000	****		
1	0	112-B1	75.0000	****		
31	5	112-B1	91.0000	****	****	
35	5	112-PD1	92.6667	****	****	
24	3	POME-PD1	94.0000	****	****	
16	2	POME-KS	94.6667	****	****	
18	2	POME-PD1	102.3333	****	****	
28	4	POME-KS	105.3333	****	****	
22	3	POME-KS	110.6667	****	****	
13	2	112-B1	112.6667	****	****	
29	4	112-PD1	125.0000	****	****	
17	2	112-PD1	127.3333	****	****	
14	2	POME-B1	130.3333	****	****	
27	4	112-KS	144.6667	****	****	****
23	3	112-PD1	147.0000	****	****	****
21	3	112-KS	198.3333	****	****	****
20	3	POME-B1	200.0000	****	****	****
38	6	POME-B1	225.0000	****	****	****
15	2	112-KS	227.0000	****	****	****
26	4	POME-B1	248.0000	****	****	****
25	4	112-B1	248.3333	****	****	****
32	5	POME-B1	330.3333		****	****
19	3	112-B1	394.6667			****



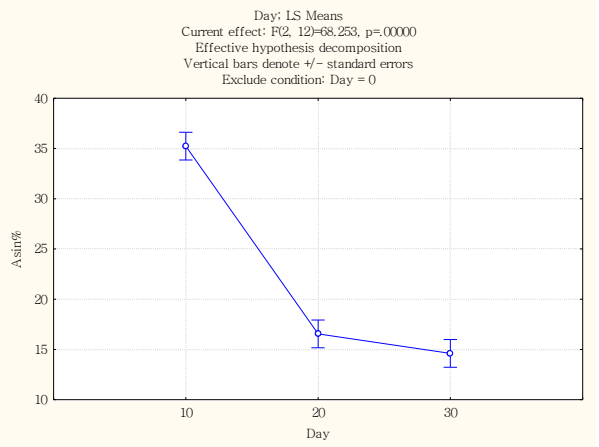
Appendix VII - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effect of live feed fed with biomass of PB grown in POME or 112 medium on survival of marble goby larvae

(i) Main effects (shaded area indicates significance at P < 0.02)

Effect	Univariate Tests of Significance for Survival (Asin%) Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Day = 0				
	SS	Degr. of Freedom	MS	F	p
Intercept	8816.268	1	8816.268	772.8733	0.000000
Day of culture	1557.137	2	778.568	68.2528	0.000000
Feed	3349.472	1	3349.472	293.6296	0.000000
Day of culture*Feed	146.398	2	73.199	6.4170	0.012730
Error	136.886	12	11.407		

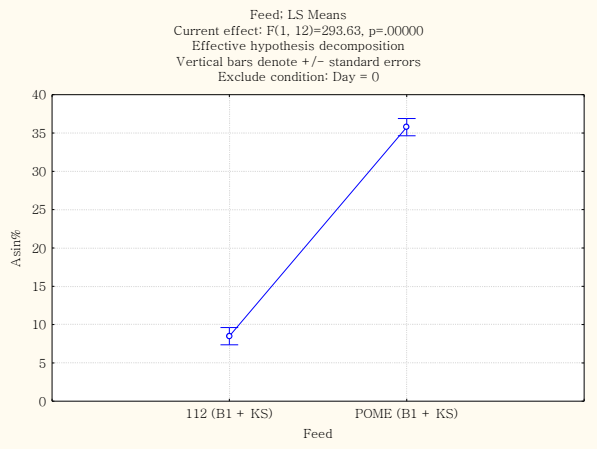
(ii) Day of culture

Cell No.	Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 11.407, df = 12.0 Exclude condition: Day = 0			
	Day	Asin% Mean	1	2
3	30	14.60815	****	
2	20	16.54865	****	
1	10	35.23698		****



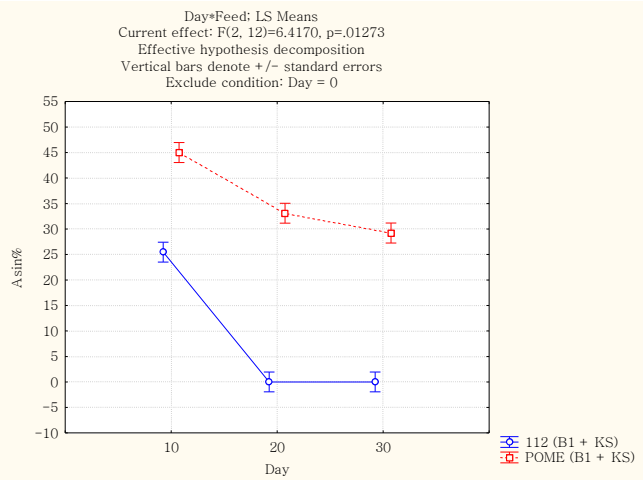
(iii) Feed

Cell No.	Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 11.407, df = 12.0 Exclude condition: Day = 0			
	Feed	Asin% Mean	1	2
1	112 (B1 + KS)	8.49008	****	
2	POME (B1 + KS)	35.77244		****



(iv) Day of culture x Feed interaction

Cell No.	Day	Asin% Mean	1	2
Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 11.407, df = 12.00(Exclude condition: Day = 0				
3	30	14.60815	****	
2	20	16.54865	****	
1	10	35.23698		****



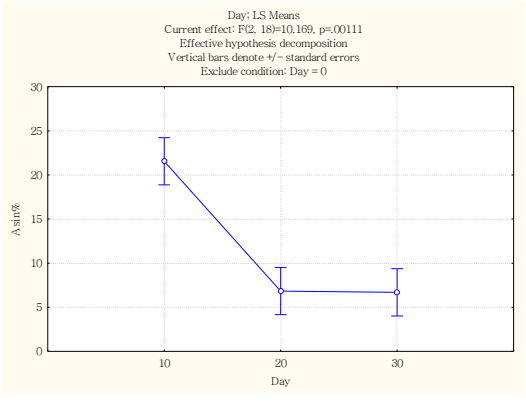
Appendix VIII - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effect of live feed fed with biomass of three species of PB grown in POME on survival of marble goby larvae

(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for Survival (Asin%) Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Day = 0					
Intercept	3694.540	1	3694.540	57.31739	0.000001
Day of culture	1310.876	2	655.438	10.16852	0.001109
Feed	2257.096	2	1128.548	17.50839	0.000060
Day of culture*Feed	29.763	4	7.441	0.11543	0.975369
Error	1160.236	18	64.458		

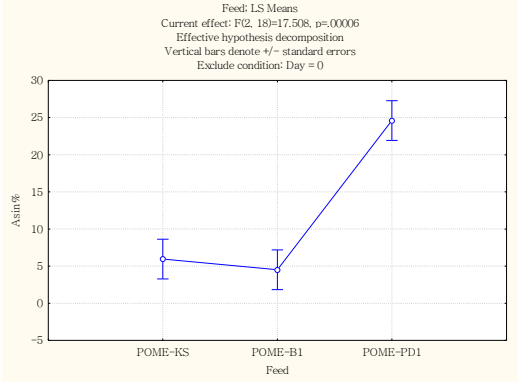
(ii) Day of culture

Cell No.	Day	Asin% Mean	1	2
Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 64.458, df = 18.00(Exclude condition: Day = 0				
3	30	6.70063	****	
2	20	6.84095	****	
1	10	21.55134		****



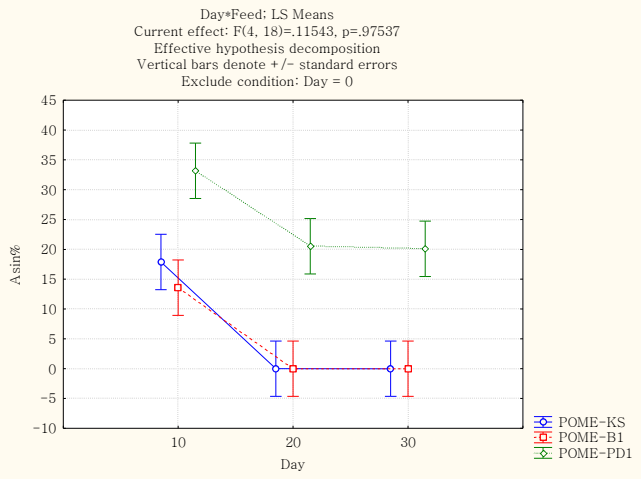
(iii) Feed

Cell No.	Feed	Asin% Mean	1	2
Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 64.458, df = 18.000 Exclude condition: Day = 0				
2	POME-B1	4.52710	****	
1	POME-KS	5.96456	****	
3	POME-PD1	24.60127		****



(iv) Day of culture x Feed interaction

Cell No.	Day	Feed	Asin% Mean	1	2
Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 64.458, df = 18.00 Exclude condition: Day = 0					
7	30	POME-KS	0.00000	****	
8	30	POME-B1	0.00000	****	
4	20	POME-KS	0.00000	****	
5	20	POME-B1	0.00000	****	
2	10	POME-B1	13.58129	****	****
1	10	POME-KS	17.89368	****	****
9	30	POME-PD1	20.10190	****	****
6	20	POME-PD1	20.52285	****	****
3	10	POME-PD1	33.17905		****



Appendix IX - Fatty acid and amino acid profiles of freeze-dried PB biomass

a) Fatty acid composition (% total fatty acids) of freeze-dried PB (B1, KS, PD1). Bacteria were cultured in either synthetic 112 medium or POME.

Structure	FAME	b112-B1	bPOME-B1	b112-KS	bPOME-KS	b112-PD1	bPOME-PD1
Saturated fatty acids							
C 4:0	Butryic	0.00000	0.00000	0.00000	0.8447	0.86637	1.19660
C 6:0	Caproic	2.40282	0.00000	8.62316	0.00000	1.53832	2.85069
C 8:0	Caprylic	0.00000	0.00000	0.20710	0.00000	0.00000	0.00000
C 10:0	Capric	0.00000	0.00000	2.68622	0.00000	0.00000	0.00000
C 11:0	Undecanoic	0.00000	0.00000	0.23581	0.00000	0.00000	0.00000
C 12:0	Lauric	14.52795	4.13301	7.67620	9.35906	38.84702	33.99657
C 13:0	Tridecanoic	0.00000	0.00000	1.01057	2.36898	0.27013	0.32871
C 14:0	Myristic	0.00000	0.00000	0.40513	2.10981	0.41062	0.00000
C 15:0	Pentadecanoic	0.00000	0.00000	0.09587	0.00000	0.00000	0.00000
C 16:0	Palmitic	0.00000	1.36931	2.83036	26.79937	1.33951	3.16018
C 17:0	Heptadecanoic	0.00000	0.00000	0.25289	0.00000	0.65564	0.59117
C 18:0	Stearic	0.00000	0.00000	2.16710	3.48022	0.81507	0.92262
C 20:0	Arachidic	0.00000	0.00000	2.01875	1.08263	2.62298	0.00000
C 21:0	Henicosanoic	0.00000	3.45788	0.00000	0.00000	5.84024	0.00000
C 22:0	Behenic	0.00000	7.38439	0.45983	0.00000	0.00000	1.25991
C 23:0	Tricosanoic	0.00000	8.15851	0.00000	0.93076	0.43636	1.11342
C 24:0	Lignoceric	0.00000	8.44802	0.38203	0.00000	0.00000	0.00000
Total		16.93077	32.95112	29.05102	46.97553	53.64226	45.41987
Monosaturated fatty acids							
C 14:1	Myristoleic	2.13092	0.00000	0.47269	1.47975	0.00000	0.42714
C 15:1	Cis-10-Pentadecenoic	1.59692	0.00000	0.64421	0.00000	0.65955	0.00000
C 16:1	Palmitoleic	0.00000	0.00000	0.19631	0.85782	0.00000	0.00000
C 17:1	Cis-10-Heptadecanoic	0.00000	0.00000	3.12553	1.99754	0.83849	0.00000

Appendix IXa, continued

Structure	FAME	b112-B1	bPOME-B1	b112-KS	bPOME-KS	b112-PD1	bPOME-PD1
C 18:1n9c	Oleic	0.00000	0.00000	1.36498	0.00000	2.46281	1.47564
C 18:1n9t	Elaidic (Trans)	49.83005	0.00000	3.14482	16.64517	0.26526	3.47446
C 20:1n9	Cis-11-Eicosenoic	0.00000	4.60271	12.46668	1.16866	4.24190	2.00768
C 22:1n9	Erucic	0.00000	0.00000	2.10002	0.00000	7.41345	0.00000
C 24:1	Nervonic	0.00000	8.15480	4.10617	13.99369	3.03820	4.36800
Total		53.55789	12.75751	27.62141	36.14263	18.91966	11.75292
Polyunsaturated fatty acids							
C 18:2n6c	Linoleic (Cis)	0.00000	0.00000	5.58511	4.66658	0.27029	0.91730
C 18:2n6t	Linolelaidic (Trans)	1.12267	0.00000	0.54158	0.00000	2.69605	0.88950
C 18:3n3	α-Linolenic	0.00000	4.48090	0.00000	2.33498	2.85312	2.22333
C 18:3n6	β-Linolenic	5.11724	2.54419	4.16896	2.58664	6.93966	16.38733
C 20:2	Cis-11,14-Eicosadienoic	0.00000	7.26493	0.33063	0.00000	2.11117	6.03049
C 20:3n3	Cis-11,14,17-Eicosatrienoic	0.00000	0.00000	1.25774	0.00000	0.00000	1.96864
C 20:3n6	Cis-8,11,14-Eicosatrienoic	9.15993	5.36878	1.55501	0.00000	0.00000	0.00000
C 20:4n6	Arachidonic (ARA)	0.00000	10.31832	0.00000	0.00000	0.00000	0.00000
C 20:5n3	Cis-5,8,11,14,17-eicosapentaenoic (EPA)	0.00000	18.01069	29.06946	1.02968	0.00000	3.93882
C 22:2	Cis-13,16 Docosadienoic	12.92150	6.30355	0.81909	6.26408	11.77225	7.87134
C 22:6n3	Cis-4,7,10,13,16,19-Docosahexaenoic (DHA)	0.00000	0.00000	0.00000	0.00000	0.79554	2.60046
Total		28.32134	54.29136	43.32758	16.88196	27.43808	42.82721

b) Amino acid composition (% protein) of freeze-dried PB (B1, KS, PD1). Bacteria were cultured in either synthetic 112 medium or POME.

Amino acid profile	b112-B1	bPOME-B1	b112-KS	bPOME-KS	b112-PD1	bPOME-PD1
Essential amino acids						
Histidine	1.732	0.994	1.764	1.121	1.205	0.901
Threonine	2.839	1.700	2.688	2.066	2.720	2.044
Valine	3.656	2.159	2.161	2.599	3.411	2.805
Methionine	1.382	0.786	3.380	0.960	1.783	1.351
Lysine	2.854	1.764	2.681	2.068	2.303	2.011
Isoleucine	2.928	1.764	2.519	2.118	2.740	2.344
Leucine	4.706	2.880	4.370	3.334	4.523	3.572
Phenylalanine	2.906	1.731	2.729	2.000	2.560	1.888
Arginine	3.439	2.001	3.261	2.523	3.635	2.648
Total	26.442	15.779	25.553	18.789	24.880	19.564

Appendix IXb, continued

Amino acid profile	b112-B1	bPOME-B1	b112-KS	bPOME-KS	b112-PD1	bPOME-PD1
Non-essential amino acids						
Aspartic acid	4.694	3.040	4.218	3.330	4.562	4.046
Serine	2.169	1.360	2.011	1.632	1.859	1.527
Glutamic acid	5.177	3.331	4.783	3.803	5.542	4.607
Glycine	3.253	1.923	3.187	2.465	2.986	2.262
Alanine	4.604	2.734	4.501	3.121	4.456	3.337
Proline	2.662	1.280	1.957	2.129	2.773	1.374
Tyrosine	1.996	1.242	2.161	1.475	1.759	1.477
Total	24.555	14.910	22.818	17.955	23.937	18.630

Chapter 4: Mass culture of *Rhodovulum sulfidophilum* grown in palm oil mill effluent for aquaculture

Appendix X - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effects of different POME concentrations and type of reactors on the production of biomass of PD1

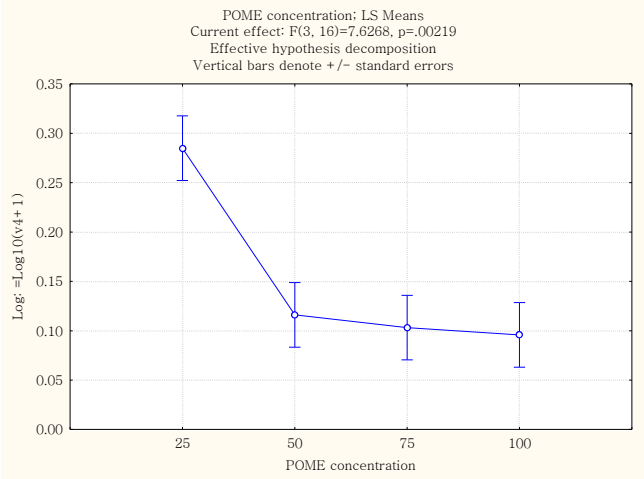
a) Dry biomass

(i) Main effects (shaded area indicates significance at P < 0.05)

Effect	Univariate Tests of Significance for Dry weight (Log g/L) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	0.540028	1	0.540028	84.14309	0.000000
% POME	0.146845	3	0.048948	7.62678	0.002186
Type of reactor	0.054354	1	0.054354	8.46905	0.010223
% POME*Type of reactor	0.065437	3	0.021812	3.39862	0.043627
Error	0.102688	16	0.006418		

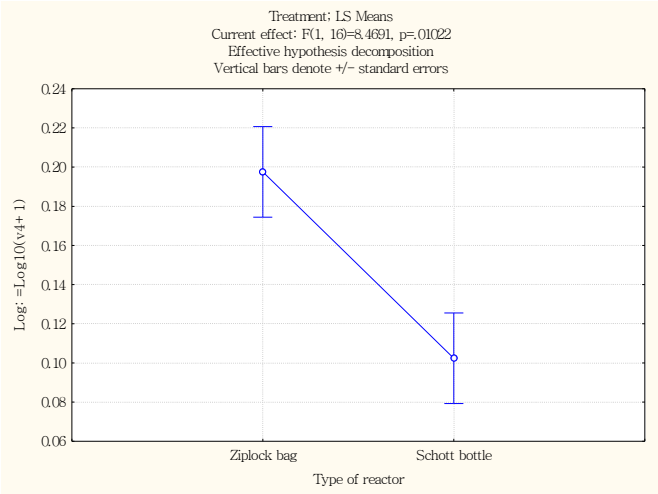
(ii) % POME

Cell No.	Tukey HSD test; variable Log Homogenous Groups, alpha = .05000 Error: Between MS = .00642, df = 16.00			
	POME concentration	Log Mean	1	2
4	100	0.095814	****	
3	75	0.103213	****	
2	50	0.116086	****	
1	25	0.284903		****



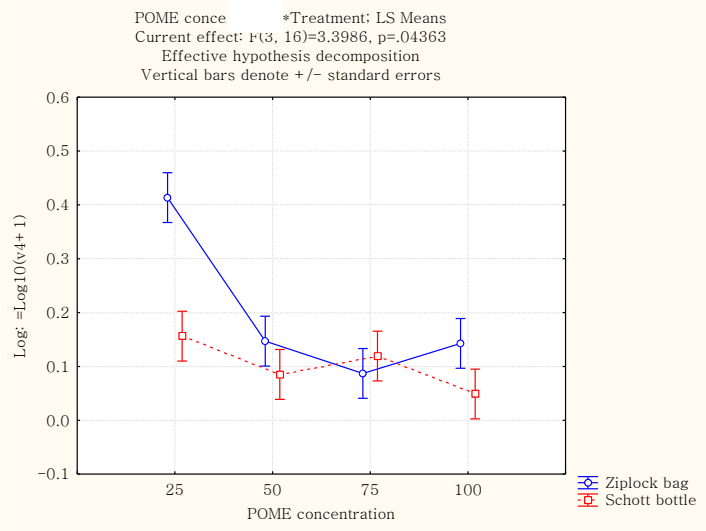
(iii) Type of reactor

Cell No.	Tukey HSD test; variable Log Homogenous Groups, alpha = .05000 Error: Between MS = .00642, df = 16.000			
	Type of reactor	Log Mean	1	2
2	Schott bottle	0.102414	****	
1	Ziplock bag	0.197593		****



(iv) % POME x Type of reactor interaction

Cell No.	POME concentration	Type of reactor	Log Mean	1	2
	Tukey HSD test; variable Log Homogenous Groups, alpha = .05000 Error: Between MS = .00642, df = 16.000				
8	100	Schott bottle	0.048825	****	
4	50	Schott bottle	0.085187	****	
5	75	Ziplock bag	0.087069	****	
6	75	Schott bottle	0.119357	****	
7	100	Ziplock bag	0.142802	****	
3	50	Ziplock bag	0.146984	****	
2	25	Schott bottle	0.156288	****	
1	25	Ziplock bag	0.413518		****



Appendix XI - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effects of different pH and type of reactors on the production of biomass and carotenoids of POME-PD1

a) Dry biomass

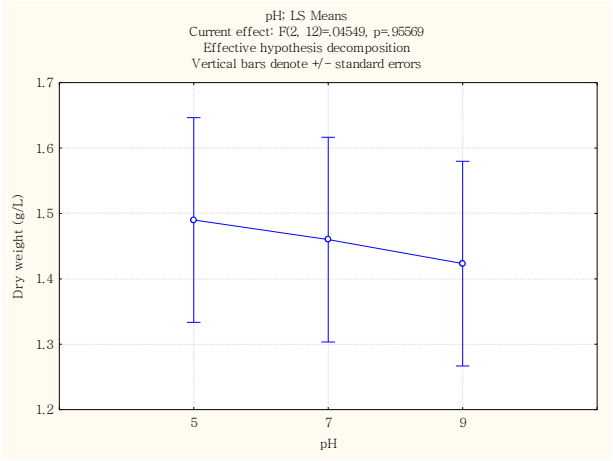
(i) Main effects

Effect	SS	Degr. of Freedom	MS	F	p
	Univariate Tests of Significance for Dry weight (g/L) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	38.25209	1	38.25209	260.1396	0.000000
pH	0.01338	2	0.00669	0.0455	0.955694
Type of reactor	0.20909	1	0.20909	1.4219	0.256131
pH*Type of reactor	0.17391	2	0.08696	0.5914	0.568930
Error	1.76453	12	0.14704		

(ii) pH

Cell No.	pH	Dry weight (g/L) Mean	1
3	9	1.423333	****
2	7	1.460000	****
1	5	1.490000	****

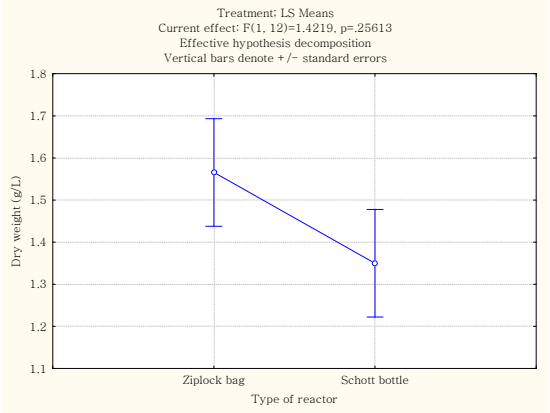
Tukey HSD test; variable Dry weight (g/L)
Homogenous Groups, alpha = .05000
Error: Between MS = .14704, df = 12.000



(iii) Type of reactor

Cell No.	Type of reactor	Dry weight (g/L) Mean	1
2	Schott bottle	1.350000	****
1	Ziplock bag	1.565556	****

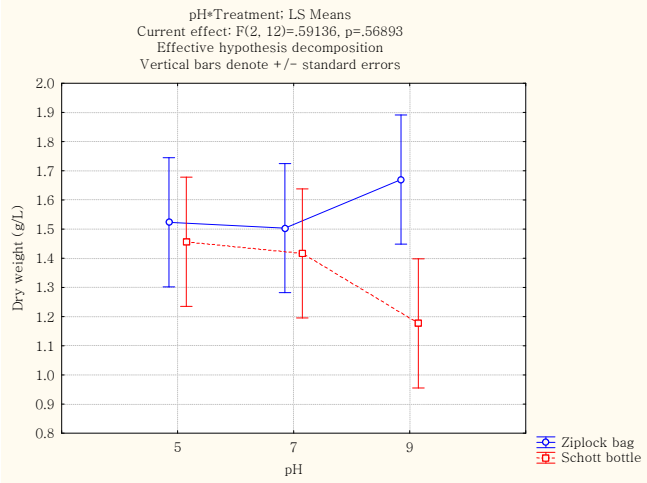
Tukey HSD test; variable Dry weight (g/L)
Homogenous Groups, alpha = .05000
Error: Between MS = .14704, df = 12.000



(iv) pH x Type of reactor interaction

Cell No.	pH	Type of reactor	Dry weight (g/L) Mean	1
6	9	Schott bottle	1.176667	****
4	7	Schott bottle	1.416667	****
2	5	Schott bottle	1.456667	****
3	7	Ziplock bag	1.503333	****
1	5	Ziplock bag	1.523333	****
5	9	Ziplock bag	1.670000	****

Tukey HSD test; variable Dry weight (g/L)
Homogenous Groups, alpha = .05000
Error: Between MS = .14704, df = 12.00



b) Total carotenoids

(i) Main effect

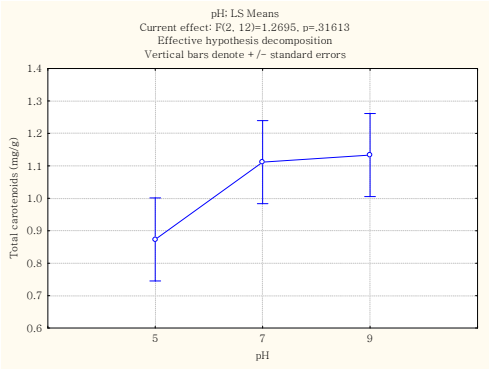
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	19.44465	1	19.44465	197.6409	0.000000
pH	0.24980	2	0.12490	1.2695	0.316130
Type of reactor	0.02267	1	0.02267	0.2305	0.639816
pH*Type of reactor	0.03705	2	0.01852	0.1883	0.830775
Error	1.18061	12	0.09838		

Univariate Tests of Significance for Total carotenoids (mg/g)
Sigma-restricted parameterization
Effective hypothesis decomposition

(ii) pH

Tukey HSD test; variable Total carotenoids (mg/g
Homogenous Groups, alpha = .05000
Error: Between MS = .09838, df = 12.000

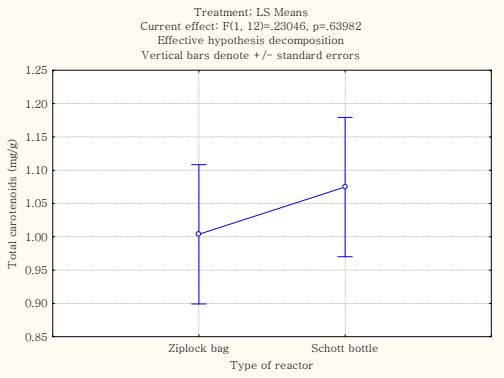
Cell No.	pH	Total carotenoids (mg/g) Mean	1
1	5	0.873237	****
2	7	1.111425	****
3	9	1.133402	****



(iii) Type of reactor

Tukey HSD test; variable Total carotenoids (mg/g
Homogenous Groups, alpha = .05000
Error: Between MS = .09838, df = 12.000

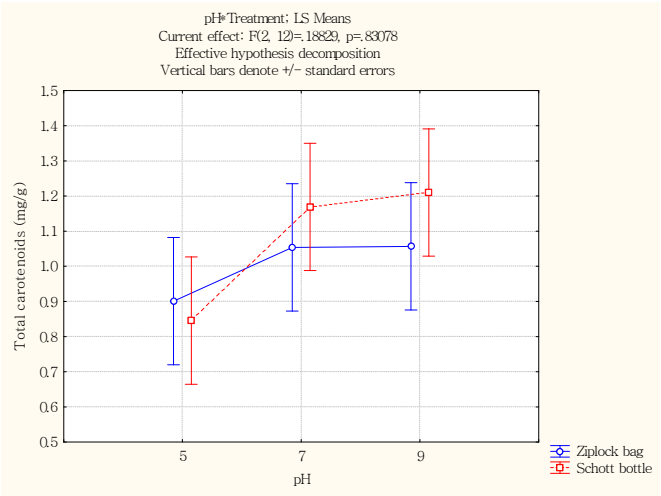
Cell No.	Type of reactor	Total carotenoids (mg/g) Mean	1
1	Ziplock bag	1.003863	****
2	Schott bottle	1.074846	****



(iv) pH x Type of reactor interaction

Tukey HSD test; variable Total carotenoids (mg/g
Homogenous Groups, alpha = .05000
Error: Between MS = .09838, df = 12.000

Cell No.	pH	Type of reactor	Total carotenoids (mg/g) Mean	1
2	5	Schott bottle	0.845518	****
1	5	Ziplock bag	0.900956	****
3	7	Ziplock bag	1.053856	****
5	9	Ziplock bag	1.056778	****
4	7	Schott bottle	1.168993	****
6	9	Schott bottle	1.210027	****



Appendix XII - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effects of different salinities and type of reactors on the production of biomass and carotenoids of POME-PD1

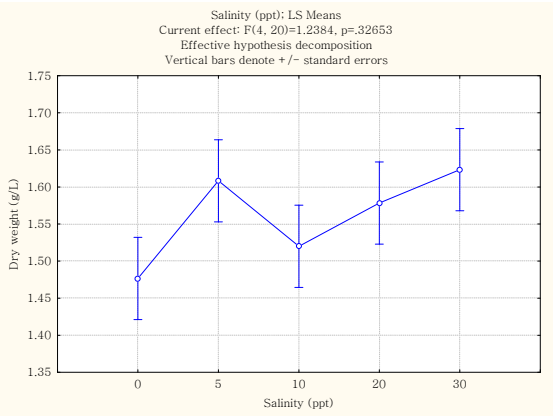
a) Dry biomass

(i) Main effects

Effect	Univariate Tests of Significance for Dry weight (g/L) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	73.13285	1	73.13285	3967.424	0.000000
Salinity	0.09131	4	0.02283	1.238	0.326525
Type of reactor	0.00161	1	0.00161	0.088	0.770400
Salinity*Type of reactor	0.08275	4	0.02069	1.122	0.373942
Error	0.36867	20	0.01843		

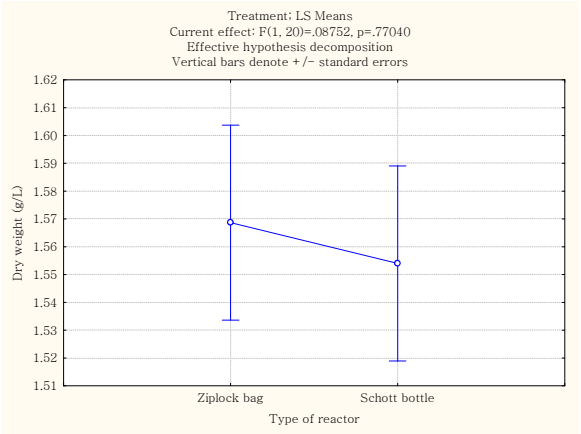
(ii) Salinity

Cell No.	Tukey HSD test; variable Dry weight (g/L) Homogenous Groups, alpha = .05000 Error: Between MS = .01843, df = 20.000		
	Salinity (ppt)	Dry weight (g/L) Mean	1
1	0	1.476667	****
3	10	1.520000	****
4	20	1.578333	****
2	5	1.608333	****
5	30	1.623333	****



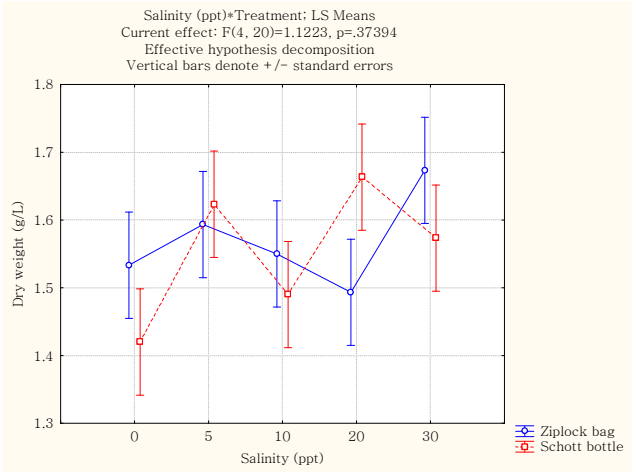
(iii) Type of reactor

Cell No.	Tukey HSD test; variable Dry weight (g/L) Homogenous Groups, alpha = .05000 Error: Between MS = .01843, df = 20.00		
	Type of reactor	Dry weight (g/L) Mean	1
2	Schott bottle	1.554000	****
1	Ziplock bag	1.568667	****



(iv) Salinity x Type of reactor interaction

Cell No.	Salinity (ppt)	Type of reactor	Dry weight (g/L) Mean	1
	Tukey HSD test; variable Dry weight (g/L) Homogenous Groups, alpha = .05000 Error: Between MS = .01843, df = 20.000			
2	0	Schott bottle	1.420000	****
6	10	Schott bottle	1.490000	****
7	20	Ziplock bag	1.493333	****
1	0	Ziplock bag	1.533333	****
5	10	Ziplock bag	1.550000	****
10	30	Schott bottle	1.573333	****
3	5	Ziplock bag	1.593333	****
4	5	Schott bottle	1.623333	****
8	20	Schott bottle	1.663333	****
9	30	Ziplock bag	1.673333	****



b) Total carotenoids

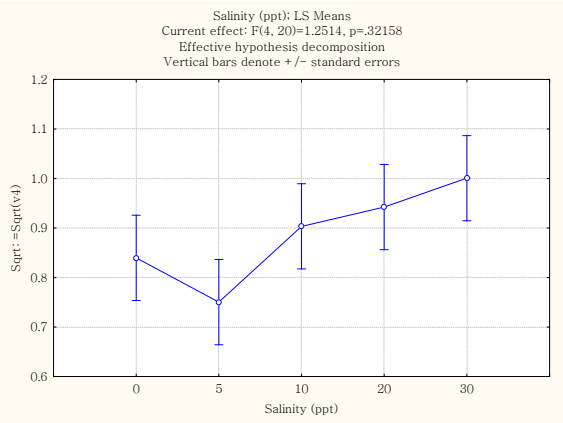
(i) Main effects (shaded area indicates significance at P < 0.03)

Effect	SS	Degr. of Freedom	MS	F	p
	Univariate Tests of Significance for Sqrt Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	23.62387	1	23.62387	530.6940	0.000000
Salinity	0.22283	4	0.05571	1.2514	0.321576
Type of reactor	1.06767	1	1.06767	23.9845	0.000087
Salinity*Type of reactor	0.61155	4	0.15289	3.4345	0.027182
Error	0.89030	20	0.04452		

(ii) Salinity

Cell No.	Salinity (ppt)	Sqrt Mean	1
2	5	0.750493	****
1	0	0.839831	****
3	10	0.903467	****
4	20	0.942414	****
5	30	1.000748	****

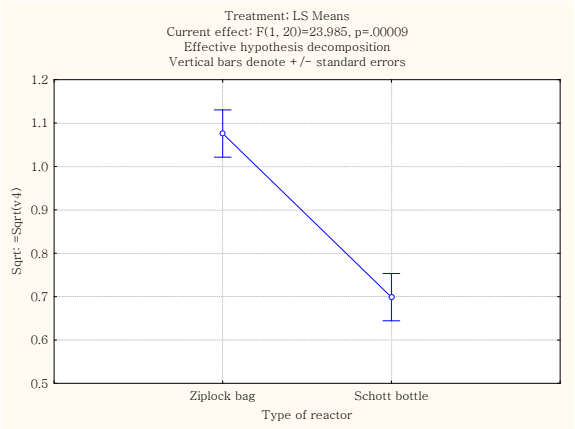
Tukey HSD test; variable Sqrt
Homogenous Groups, alpha = .05000
Error: Between MS = .04452, df = 20.000



(iii) Type of reactor

Cell No.	Type of reactor	Sqrt Mean	1	2
2	Schott bottle	0.698740	****	
1	Ziplock bag	1.076041		****

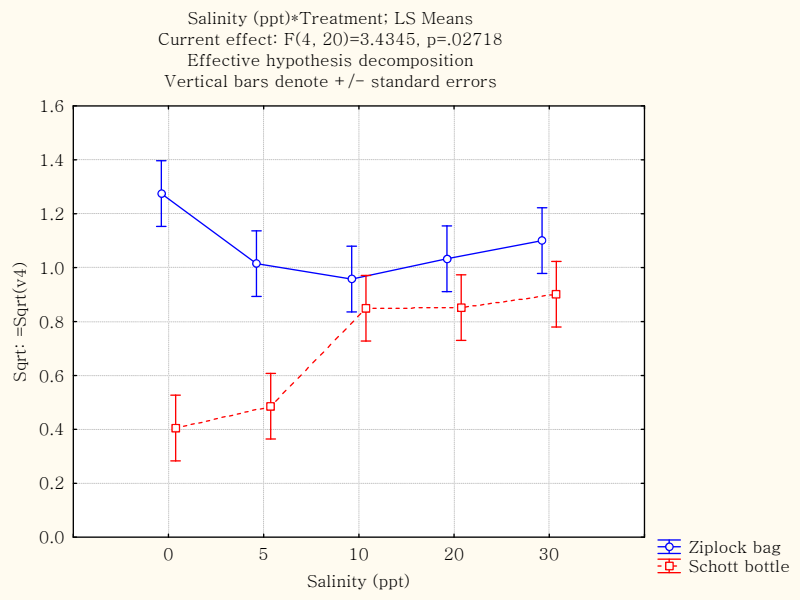
Tukey HSD test; variable Sqrt
Homogenous Groups, alpha = .05000
Error: Between MS = .04452, df = 20.00



(iv) Salinity x Type of reactor interaction

Cell No.	Salinity (ppt)	Type of reactor	Sqrt Mean	1	2	3
2	0	Schott bottle	0.405116		****	
4	5	Schott bottle	0.486173		****	****
6	10	Schott bottle	0.849242	****	****	****
8	20	Schott bottle	0.851838	****	****	****
10	30	Schott bottle	0.901333	****	****	****
5	10	Ziplock bag	0.957693	****	****	****
3	5	Ziplock bag	1.014814	****	****	****
7	20	Ziplock bag	1.032991	****		****
9	30	Ziplock bag	1.100164	****		
1	0	Ziplock bag	1.274546	****		

Tukey HSD test; variable Sqrt
Homogenous Groups, alpha = .05000
Error: Between MS = .04452, df = 20.000



Appendix XIII - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effects of different light intensities and type of reactors on the production of biomass and carotenoids of POME-PD1

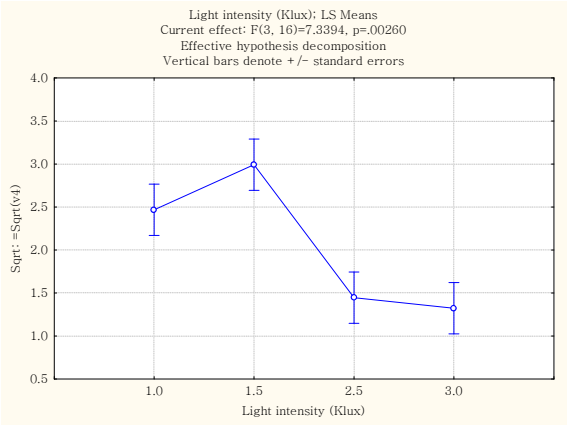
a) Dry biomass

(i) Main effects (shaded area indicates significance at P < 0.04)

Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for Dry weight (Sqrt g/L Sigma-restricted parameterization Effective hypothesis decomposition					
Intercept	101.6201	1	101.6201	190.6652	0.000000
Klux	11.7352	3	3.9117	7.3394	0.002598
Type of reactor	2.6897	1	2.6897	5.0467	0.039142
Klux*Type of reactor	1.9074	3	0.6358	1.1929	0.343996
Error	8.5276	16	0.5330		

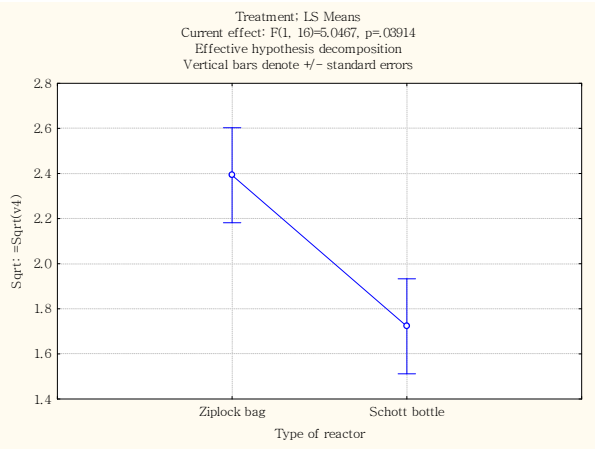
(ii) Light intensity (Klux)

Cell No.	Light intensity	Sqrt Mean	1	2
Tukey HSD test; variable Sqrt Homogenous Groups, alpha = .05000 Error: Between MS = .53298, df = 16.000				
4	3.0	1.323649	****	
3	2.5	1.446134	****	
1	1.0	2.468203	****	****
2	1.5	2.992853		****



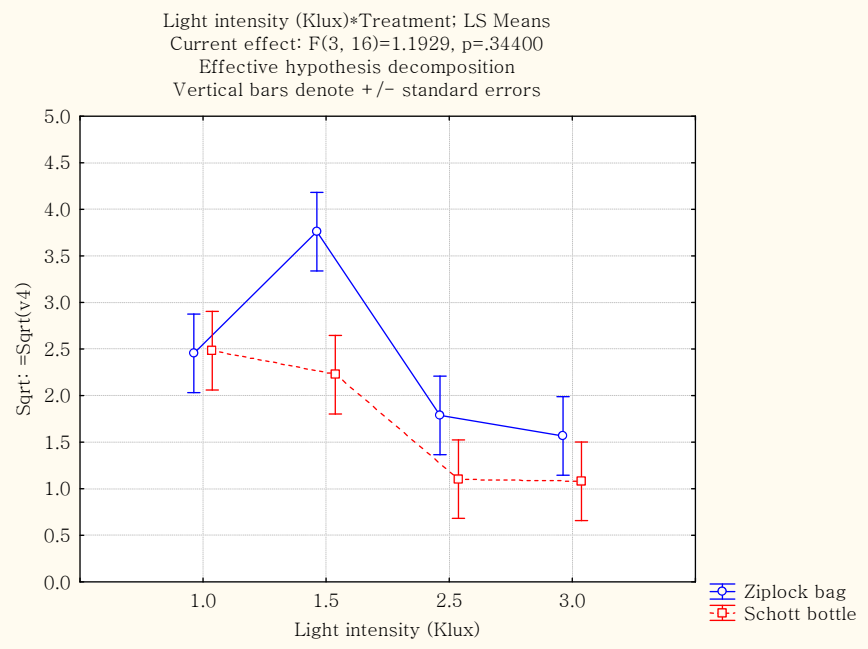
(iii) Type of reactor

Cell No.	Type of reactor	Sqrt Mean	1	2
Tukey HSD test; variable Sqrt Homogenous Groups, alpha = .05000 Error: Between MS = .53298, df = 16.000				
2	Schott bottle	1.722937	****	
1	Ziplock bag	2.392483		****



(iv) Light intensity (Klux) x Type of reactor interaction

Cell No.	Light intensity	Type of reactor	Sqrt Mean	1	2
Tukey HSD test; variable Sqrt Homogenous Groups, alpha = .05000 Error: Between MS = .53298, df = 16.000					
8	3.0	Schott bottle	1.080227	****	
6	2.5	Schott bottle	1.104056	****	
7	3.0	Ziplock bag	1.567072	****	
5	2.5	Ziplock bag	1.788211	****	****
4	1.5	Schott bottle	2.224930	****	****
1	1.0	Ziplock bag	2.453871	****	****
2	1.0	Schott bottle	2.482535	****	****
3	1.5	Ziplock bag	3.760776		****



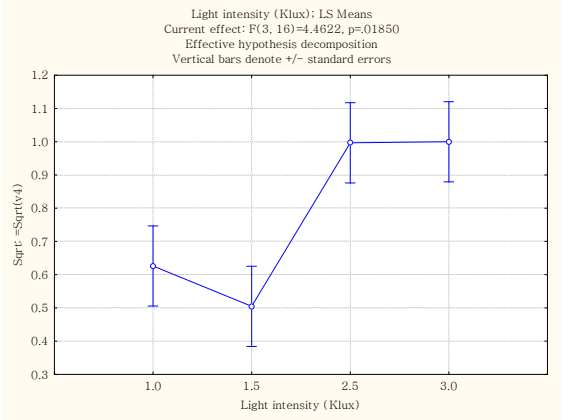
b) Total carotenoids

(i) Main effects (shaded area indicates significance at P < 0.02)

Effect	Univariate Tests of Significance for Total carotenoids (Sqrt mg/g) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	14.67773	1	14.67773	168.1224	0.000000
Klux	1.16869	3	0.38956	4.4622	0.018497
Type of reactor	0.25413	1	0.25413	2.9108	0.107318
Klux*Type of reactor	0.38194	3	0.12731	1.4583	0.263328
Error	1.39686	16	0.08730		

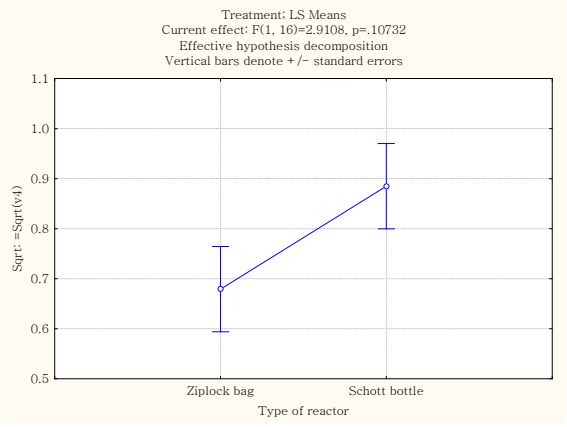
(ii) Light intensity (Klux)

Cell No.	Tukey HSD test; variable Sqrt Homogenous Groups, alpha = .05000 Error: Between MS = .08730, df = 16.000 Light intensity	Sqrt Mean	1	2
2	1.5	0.504834		****
1	1.0	0.626336	****	****
3	2.5	0.996844	****	
4	3.0	1.000110	****	



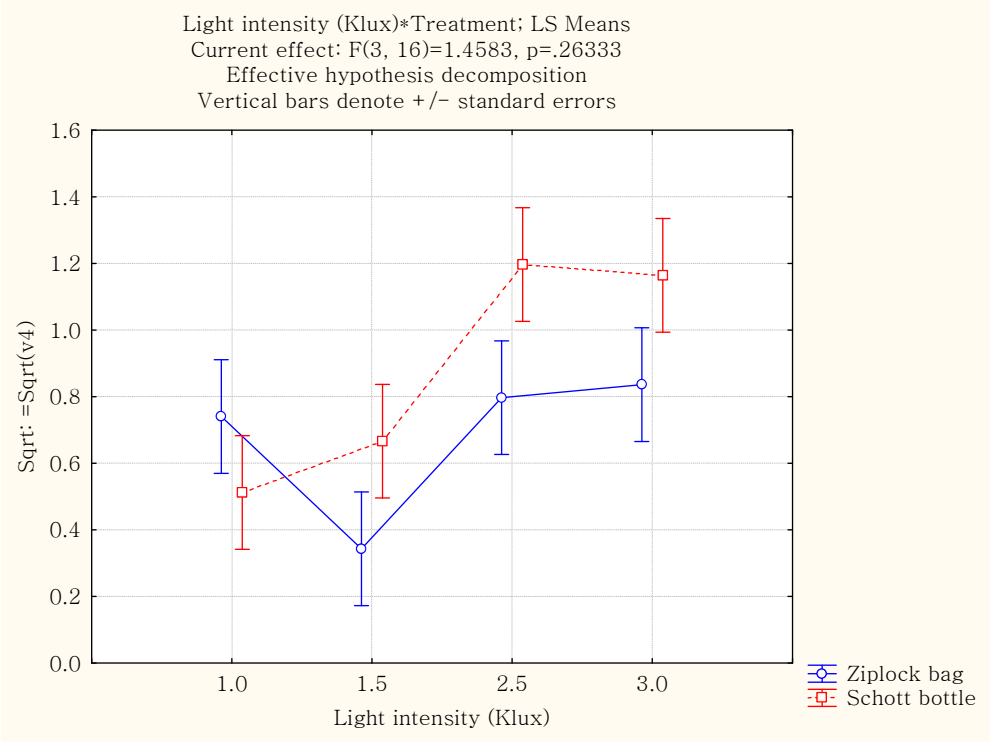
(iii) Type of reactor

Cell No.	Tukey HSD test; variable Sqrt Homogenous Groups, alpha = .05000 Error: Between MS = .08730, df = 16.00 Type of reactor	Sqrt Mean	1
1	Ziplock bag	0.679130	****
2	Schott bottle	0.884932	****



(iv) Light intensity (Klux) x Type of reactor interaction

Cell No.	Tukey HSD test; variable Sqrt Homogenous Groups, alpha = .05000 Error: Between MS = .08730, df = 16.000 Light intensity	Type of reactor	Sqrt Mean	1	2
3	1.5	Ziplock bag	0.343202	****	
2	1.0	Schott bottle	0.512314	****	****
4	1.5	Schott bottle	0.666466	****	****
1	1.0	Ziplock bag	0.740358	****	****
5	2.5	Ziplock bag	0.796959	****	****
7	3.0	Ziplock bag	0.836001	****	****
8	3.0	Schott bottle	1.164219	****	****
6	2.5	Schott bottle	1.196729		****

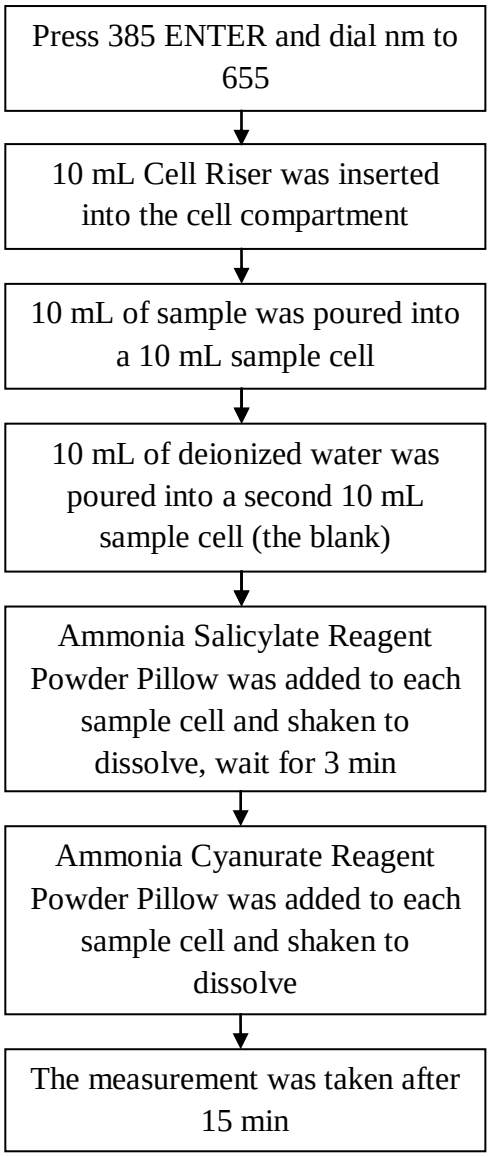

Light intensity (Klux)*Treatment; LS Means
Current effect: F(3, 16)=1.4583, p=.26333
Effective hypothesis decomposition
Vertical bars denote +/- standard errors
Sqrt: =Sqrt(v4)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.0
1.5
2.5
3.0
Light intensity (Klux)
Ziplock bag
Schott bottle

Appendix XIV - Methodology of nutrient analysis (HACH water analysis handbook, 1997)

Parameter	Method	Detection Range	Wavelength	Reagent	Summary of Method
a) Nitrogen, Ammonia, low range, (NH_3-N) (method 8155)	Salicylate method	0.00 to 0.50 mg/L	655 nm	Ammonia salicylate reagent powder pillow Ammonia cyanurate reagent powder pillow	Ammonia compounds combine with chlorine to form monochloramine. Monochloramine reacts with salicylate to form 5 - aminosalicylate. The 5-aminosalicylate is oxidized in the presence of a sodium nitroprusside catalyst to form a blue coloured compound. The blue colour is masked by the yellow colour from the excess reagent present to give a final green-coloured solution.
b) Nitrite, low range, (NO_2-N) (Method 8507)	Diazotization method	0.00 to 0.30 mg/L	507 nm	NitriVer 3 nitrite reagent powder pillow	Nitrite in the sample reacts with sulfanilamide to form an intermediate diazonium salt. This couples with N – (1-napthyl)-ethylenediamine dihydrochloride to produce a red-coloured complex directly proportional to the amount of nitrite present.
c) Nitrate, low range, (NO_3-N) (Method 8192)	Cadmium reduction method	0.00 to 0.40 mg/L	507 nm	NitraVer 6 nitrate reagent powder pillow NitriVer 3 nitrite reagent powder pillow	Cadmium metal reduces nitrates present in the sample to nitrite. The nitrite ion reacts in an acidic medium with sulfanilic acid to form an intermediate diazonium salt which couples to chromotropic acid to from a pink-coloured product.

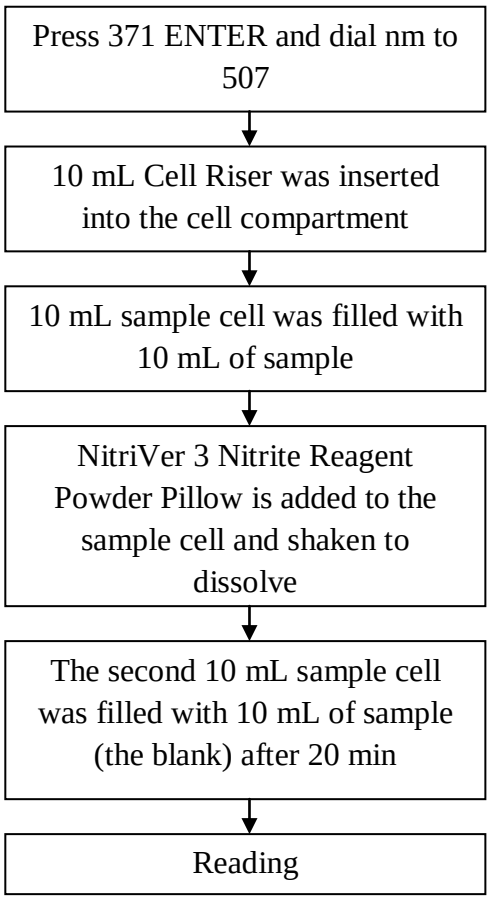
Appendix XIVa - Nitrogen, Ammonia, low range (0 to 0.50 mg/L NH_3-N) Method 8155

The nitrogen, ammonia was determined based on salicylate method using a spectrophotometer DR/2010. The sample was filtered through 47 mm Whatman glass mircofibre filter paper prior to analysis. The method is as follows:



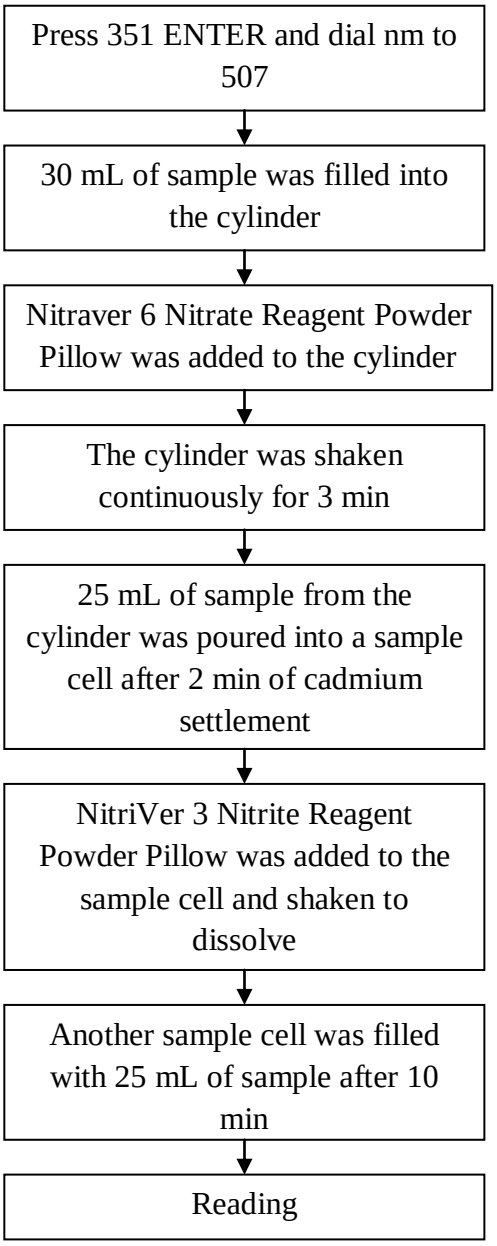
Appendix XIVb - Nitrite, low range (0-0.300mg/L NO_2-N) (Method 8507)

The nitrite was determined based on diazotization method using a spectrophotometer DR/2010. The sample was filtered through 47 mm Whatman glass mircofibre filter paper prior to analysis. The method is as follows:



Appendix XIVc - Nitrate, low range (0 to 0.40 mg/L NO_3-N) (Method 8192)

The nitrate was determined based on cadmium reduction method using a spectrophotometer DR/2010. The sample was filtered through 47 mm Whatman glass mircofibre filter paper prior to analysis. The method is as follows:



Chapter 5: Phototrophic bacteria grown in palm oil mill effluent as a total feed for the rotifer, *Brachionus rotundiformis*

Appendix XV – Summary results of Kruskal-Wallis test on the production of rotifers fed with bacterial diet compared to microalgal diet (Experiment 1)

a) Rotifer density

(i) Main effect

Kruskal-Wallis ANOVA by Ranks; ind/mL
Independent (grouping) variable: Feed
Kruskal-Wallis test: H (3, N= 12) =4.846154 p =.183

Depend.: ind/ml	Code	Valid N	Sum of Ranks
bPOME-PD1	101	3	27.00000
Oven dried bPOME-PD1	102	3	9.00000
cPOME-PD1	103	3	18.00000
Nanno	104	3	24.00000

Median Test, Overall Median = 197.000; ind/mL
Independent (grouping) variable: Feed
Chi-Square = 6.666667 df = 3 p = .0833

Dependent: ind/ml		bPOME-PD1	Oven dried bPOME-PD1	cPOME-PD1	Nanno	Total
<= Median:	observed	0.00000	3.00000	2.000000	1.000000	6.00000
	expected	1.50000	1.50000	1.500000	1.500000	
	obs.-exp.	-1.50000	1.50000	0.500000	-0.500000	
> Median:	observed	3.00000	0.00000	1.000000	2.000000	6.00000
	expected	1.50000	1.50000	1.500000	1.500000	
	obs.-exp.	1.50000	-1.50000	-0.500000	0.500000	
Total:	observed	3.00000	3.00000	3.000000	3.000000	12.00000

b) Growth rate

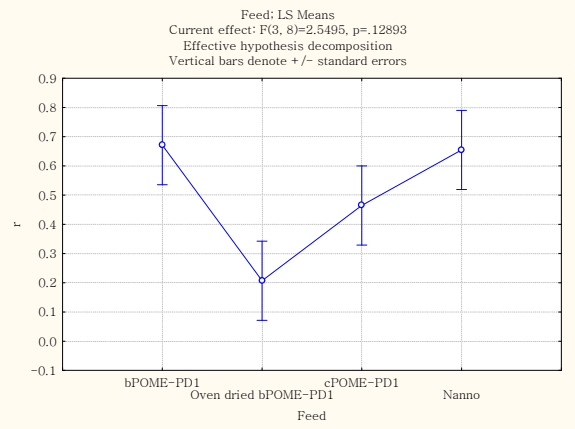
(i) Main effect

Univariate Tests of Significance for growth rate
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	2.993437	1	2.993437	54.40330	0.000078
Feed	0.420843	3	0.140281	2.54949	0.128928
Error	0.440185	8	0.055023		

(ii) Feed

Cell No.	Feed	r Mean	1
Tukey HSD test; variable r Homogenous Groups, alpha = .05000 Error: Between MS = .05502, df = 8.000(			
2	Oven dried bPOME-PD	0.207093	****
3	cPOME-PD1	0.464796	****
4	Nanno	0.654729	****
1	bPOME-PD1	0.671193	****



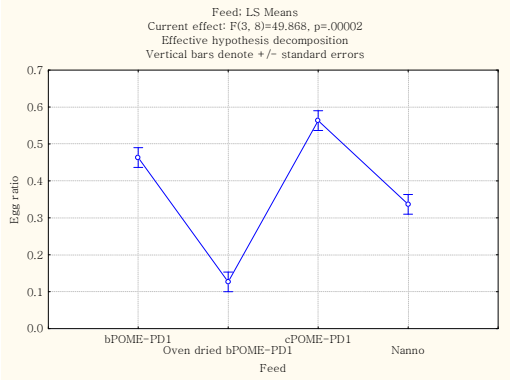
c) Egg ratio

(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for Egg ratio Sigma-restricted parameterization Effective hypothesis decomposition					
Intercept	1.665075	1	1.665075	780.5039	0.000000
Feed	0.319158	3	0.106386	49.8685	0.000016
Error	0.017067	8	0.002133		

(ii) Feed

Cell No.	Feed	Egg ratio Mean	1	2	3
Tukey HSD test; variable Egg ratio Homogenous Groups, alpha = .05000 Error: Between MS = .00213, df = 8.0000					
2	Oven dried bPOME-PD1	0.126667		****	
4	Nanno	0.336667			****
1	bPOME-PD1	0.463333	****		
3	cPOME-PD1	0.563333	****		



Appendix XVI - Summary results of two-way ANOVA and posthoc Tukey HSD test on the production of rotifers fed with different rations of bacterial diet (Experiment 2)

a) Rotifer density

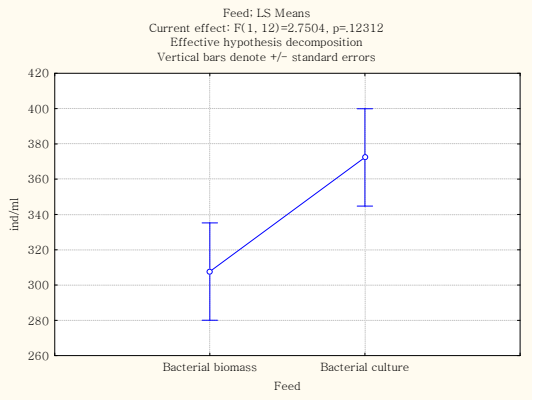
(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for ind/mL Sigma-restricted parameterization Effective hypothesis decomposition					
Intercept	2080800	1	2080800	304.1216	0.000000
Feed	18818	1	18818	2.7504	0.123122
Ration	270703	2	135352	19.7824	0.000159
Feed*Ration	284551	2	142275	20.7944	0.000126
Error	82104	12	6842		

(ii) Feed

Tukey HSD test; variable ind/ml
Homogenous Groups, alpha = .05000
Error: Between MS = 6842.0, df = 12.00

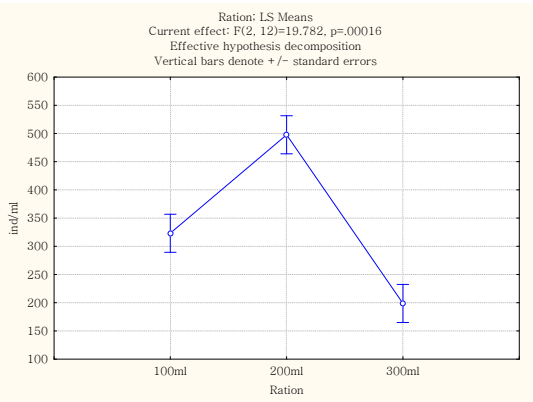
Cell No.	Feed	ind/ml Mean	1
1	Bacterial biomas	307.6667	****
2	Bacterial cultur	372.3333	****



(iii) Ration

Tukey HSD test; variable ind/ml
Homogenous Groups, alpha = .05000
Error: Between MS = 6842.0, df = 12.00

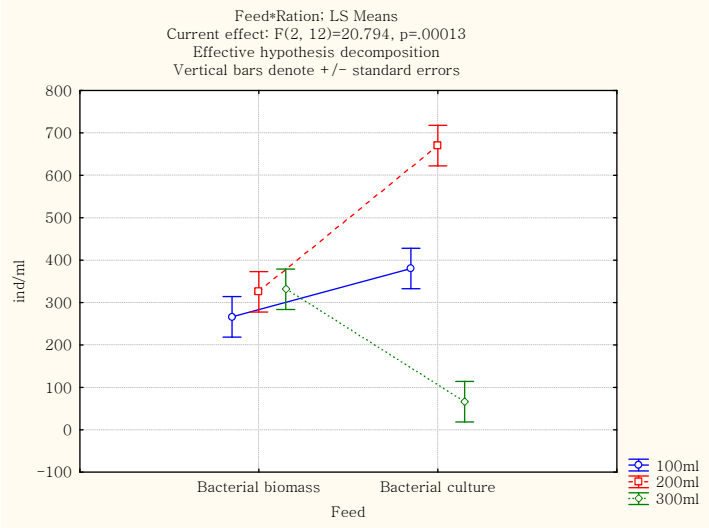
Cell No.	Ration	ind/ml Mean	1	2
3	300ml	198.8333	****	
1	100ml	323.3333	****	
2	200ml	497.8333		****



(iv) Feed x Ration interaction

Tukey HSD test; variable ind/ml
Homogenous Groups, alpha = .05000
Error: Between MS = 6842.0, df = 12.000

Cell No.	Feed	Ration	ind/ml Mean	1	2	3
6	Bacterial culture	300ml	66.3333		****	
1	Bacterial biomass	100ml	266.3333	****	****	
2	Bacterial biomass	200ml	325.3333	****		
3	Bacterial biomass	300ml	331.3333	****		
4	Bacterial culture	100ml	380.3333	****		
5	Bacterial culture	200ml	670.3333			****



b) Growth rate

(i) Main effects (shaded area indicates significance at P < 0.01)

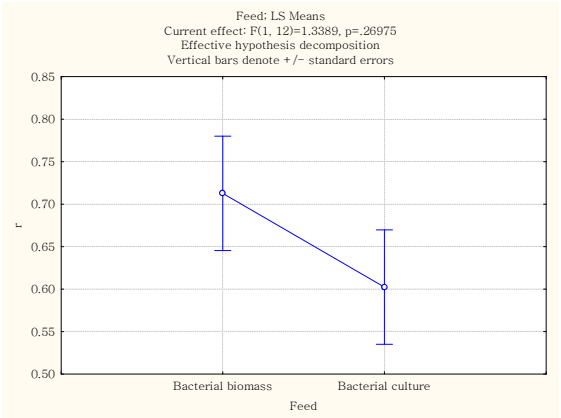
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	7.783472	1	7.783472	190.6509	0.000000
Feed	0.054660	1	0.054660	1.3389	0.269749
Ration	2.519069	2	1.259534	30.8514	0.000019
Feed*Ration	0.662350	2	0.331175	8.1119	0.005907
Error	0.489909	12	0.040826		

Univariate Tests of Significance for growth rate
Sigma-restricted parameterization
Effective hypothesis decomposition

(ii) Feed

Cell No.	Feed	r Mean	1
2	Bacterial culture	0.602477	****
1	Bacterial biomass	0.712689	****

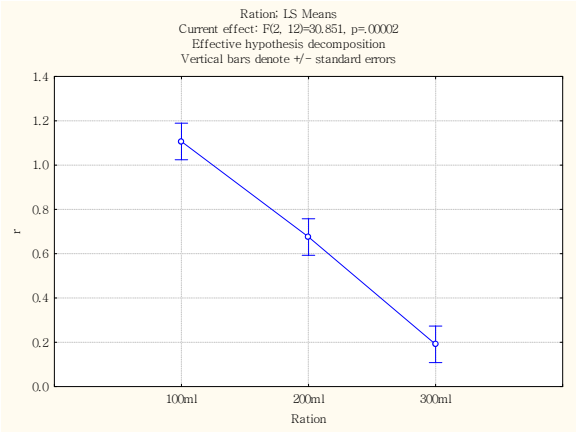
Tukey HSD test; variable r
Homogenous Groups, alpha = .05000
Error: Between MS = .04083, df = 12.000



(iii) Ration

Cell No.	Ration	r Mean	1	2	3
3	300ml	0.190744	****		
2	200ml	0.675438		****	
1	100ml	1.106567			****

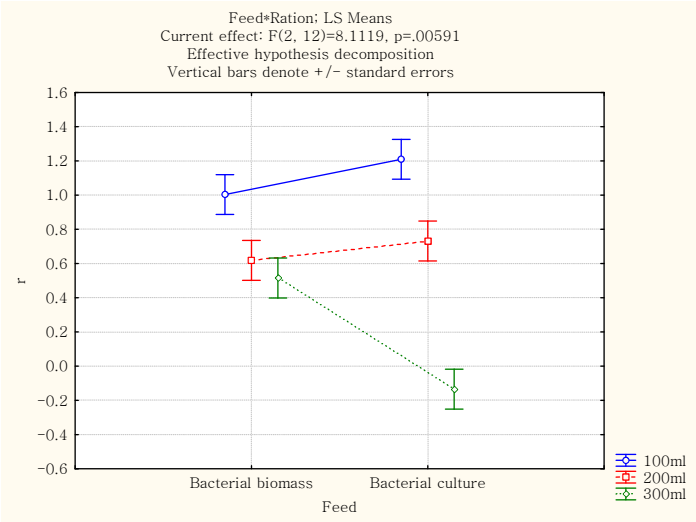
Tukey HSD test; variable r
Homogenous Groups, alpha = .05000
Error: Between MS = .04083, df = 12.000



(iv) Feed x Ration interaction

Cell No.	Feed	Ration	r Mean	1	2	3
6	Bacterial culture	300ml	-0.134315			****
3	Bacterial biomass	300ml	0.515802	****		
2	Bacterial biomass	200ml	0.618810	****		
5	Bacterial culture	200ml	0.732065	****	****	
1	Bacterial biomass	100ml	1.003455	****	****	
4	Bacterial culture	100ml	1.209680		****	

Tukey HSD test; variable r
Homogenous Groups, alpha = .05000
Error: Between MS = .04083, df = 12.000



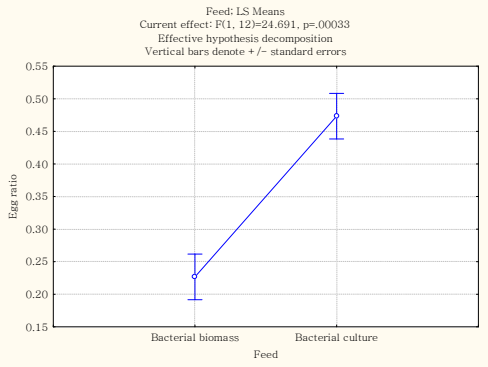
c) Egg ratio

(i) Main effects (shaded area indicates significance at P < 0.02)

Effect	SS	Degr. of Freedom	MS	F	p
	Univariate Tests of Significance for Egg ratio Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	2.205000	1	2.205000	198.8477	0.000000
Feed	0.273800	1	0.273800	24.6914	0.000326
Ration	0.148900	2	0.074450	6.7139	0.011047
Feed*Ration	0.082233	2	0.041117	3.7079	0.055738
Error	0.133067	12	0.011089		

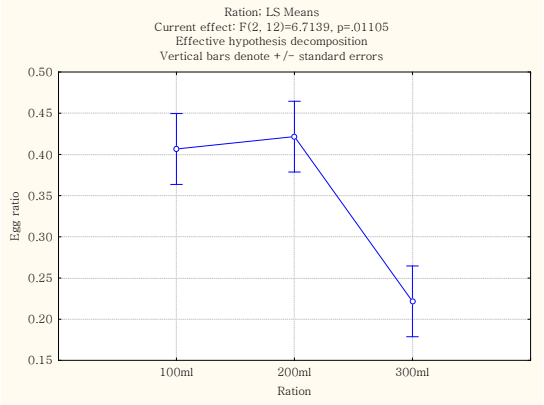
(ii) Feed

Cell No.	Feed	Egg ratio Mean	1	2
	Tukey HSD test; variable Egg ratio Homogenous Groups, alpha = .05000 Error: Between MS = .01109, df = 12.000			
1	Bacterial biomass	0.226667	****	
2	Bacterial culture	0.473333		****



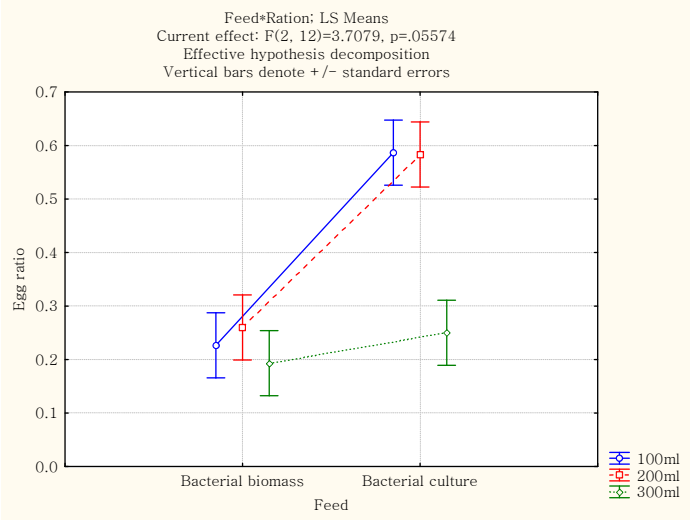
(iii) Ration

Cell No.	Ration	Egg ratio Mean	1	2
	Tukey HSD test; variable Egg ratio Homogenous Groups, alpha = .05000 Error: Between MS = .01109, df = 12.00			
3	300ml	0.221667		****
1	100ml	0.406667	****	
2	200ml	0.421667	****	



(iv) Feed x Ration interaction

Cell No.	Feed	Ration	Egg ratio Mean	1	2
Tukey HSD test; variable Egg ratio Homogenous Groups, alpha = .05000 Error: Between MS = .01109, df = 12.000					
3	Bacterial biomass	300ml	0.193333	****	
1	Bacterial biomass	100ml	0.226667	****	
6	Bacterial culture	300ml	0.250000	****	
2	Bacterial biomass	200ml	0.260000	****	
5	Bacterial culture	200ml	0.583333		****
4	Bacterial culture	100ml	0.586667		****



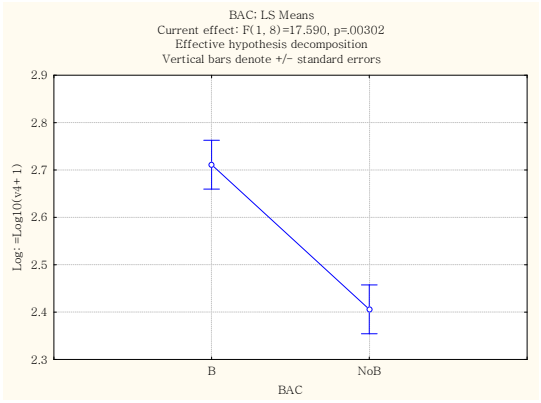
Appendix XVII - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effects of bacteria and POME on rotifer production (Experiment 3)

a) Rotifer density

(i) Main effects (shaded area indicates significance at P < 0.01)

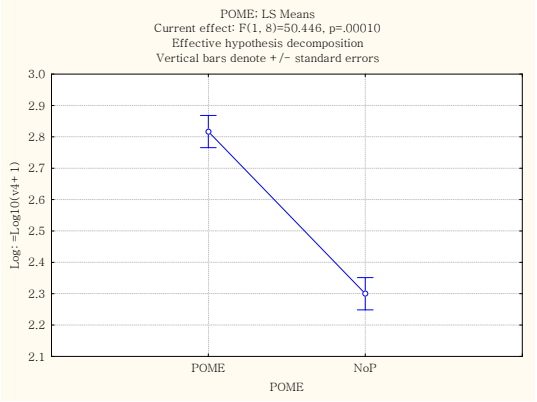
Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for Log Sigma-restricted parameterization Effective hypothesis decomposition					
Intercept	78.56028	1	78.56028	4942.197	0.000000
BAC	0.27960	1	0.27960	17.590	0.003021
POME	0.80189	1	0.80189	50.446	0.000102
BAC*POME	0.03606	1	0.03606	2.268	0.170455
Error	0.12717	8	0.01590		

(ii) Bacteria



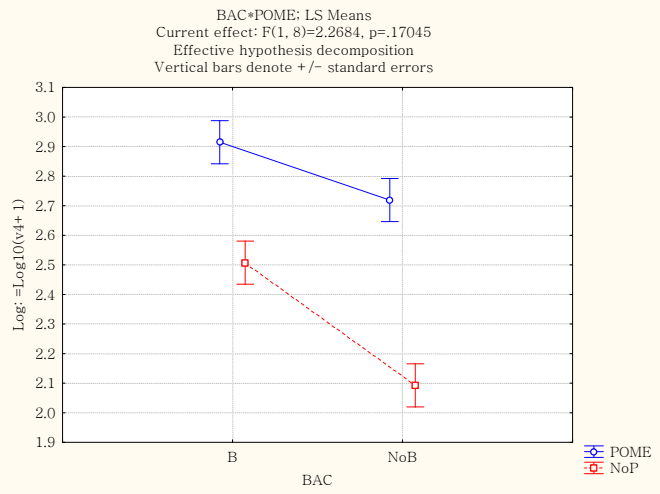
Cell No.	BAC	Log Mean	1	2
Tukey HSD test; variable Log Homogenous Groups, alpha = .05000 Error: Between MS = .01590, df = 8.00(				
2	NoB	2.406006	****	
1	B	2.711294		****

(iii) POME



Cell No.	POME	Log Mean	1	2
Tukey HSD test; variable Log Homogenous Groups, alpha = .05000 Error: Between MS = .01590, df = 8.00(				
2	NoP	2.300147	****	
1	POME	2.817153		****

(iv) Bacteria x POME interaction



Cell No.	BAC	Log Mean	1	2
Tukey HSD test; variable Log Homogenous Groups, alpha = .05000 Error: Between MS = .01590, df = 8.00				
2	NoB	2.406006	****	
1	B	2.711294		****

b) Growth rate

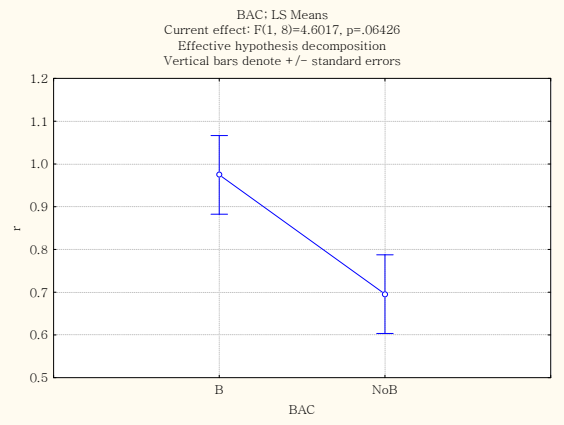
(i) Main effects

Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for growth rate Sigma-restricted parameterization Effective hypothesis decomposition					
Intercept	8.368700	1	8.368700	164.9698	0.000001
BAC	0.233441	1	0.233441	4.6017	0.064257
POME	0.147483	1	0.147483	2.9073	0.126576
BAC*POME	0.101102	1	0.101102	1.9930	0.195719
Error	0.405829	8	0.050729		

(ii) Bacteria

Cell No.	BAC	r Mean	1
2	NoB	0.695624	****
1	B	0.974575	****

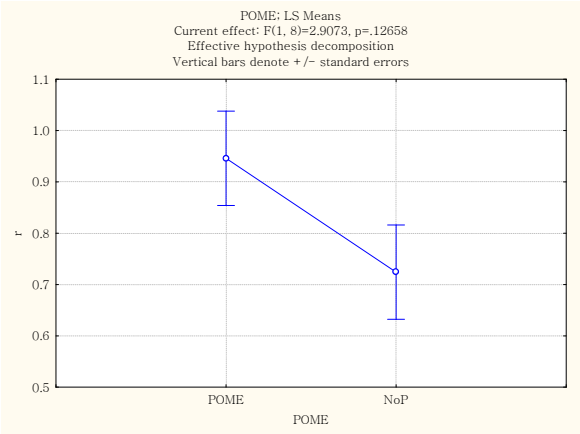
Tukey HSD test; variable r
Homogenous Groups, alpha = .05000
Error: Between MS = .05073, df = 8.000(



(iii) POME

Cell No.	POME	r Mean	1
2	NoP	0.724239	****
1	POME	0.945961	****

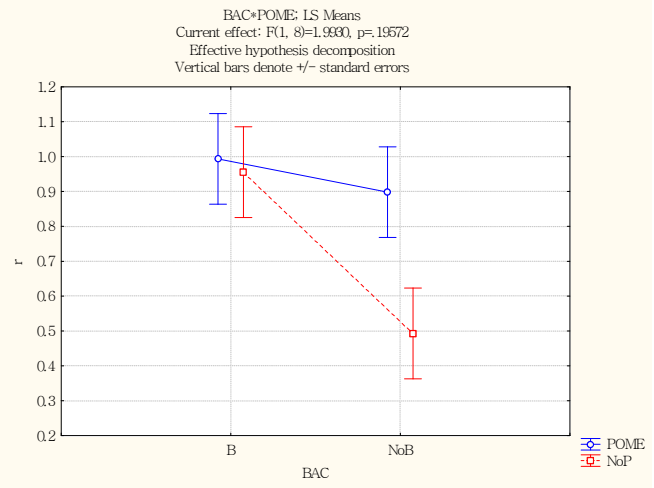
Tukey HSD test; variable r
Homogenous Groups, alpha = .05000
Error: Between MS = .05073, df = 8.0000



(iv) Bacteria x POME interaction

Cell No.	BAC	POME	r Mean	1
4	NoB	NoP	0.492974	****
3	NoB	POME	0.898274	****
2	B	NoP	0.955503	****
1	B	POME	0.993648	****

Tukey HSD test; variable r
Homogenous Groups, alpha = .05000
Error: Between MS = .05073, df = 8.0000



c) Egg ratio

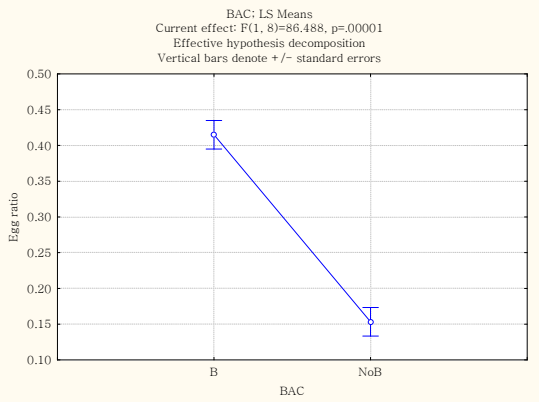
(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.969008	1	0.969008	408.0035	0.000000
BAC	0.205408	1	0.205408	86.4877	0.000015
POME	0.232408	1	0.232408	97.8561	0.000009
BAC*POME	0.000075	1	0.000075	0.0316	0.863372
Error	0.019000	8	0.002375		

Univariate Tests of Significance for Egg ratio
Sigma-restricted parameterization
Effective hypothesis decomposition

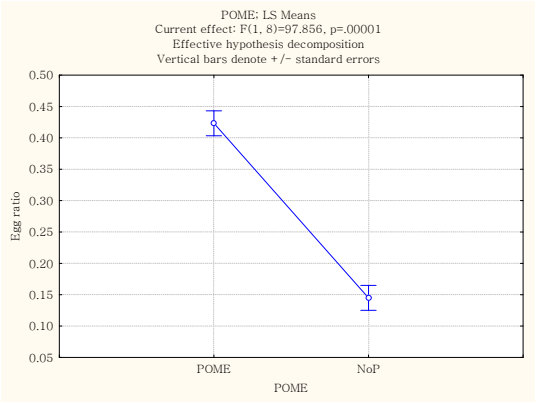
(ii) Bacteria

Cell No.	BAC	Egg ratio Mean	1	2
Tukey HSD test; variable Egg ratio Homogenous Groups, alpha = .05000 Error: Between MS = .00237, df = 8.0000				
2	NoB	0.153333	****	
1	B	0.415000		****



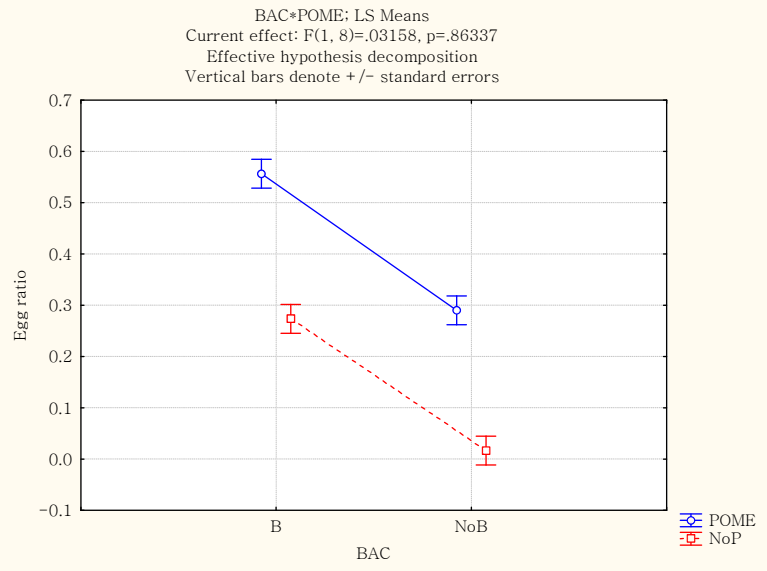
(iii) POME

Cell No.	POME	Egg ratio Mean	1	2
Tukey HSD test; variable Egg ratio Homogenous Groups, alpha = .05000 Error: Between MS = .00237, df = 8.00(				
2	NoP	0.145000	****	
1	POME	0.423333		****



(iv) Bacteria x POME interaction

Cell No.	BAC	POME	Egg ratio Mean	1	2	3
Tukey HSD test; variable Egg ratio Homogenous Groups, alpha = .05000 Error: Between MS = .00237, df = 8.0000						
4	NoB	NoP	0.016667		****	
2	B	NoP	0.273333	****		
3	NoB	POME	0.290000	****		
1	B	POME	0.556667			****



Appendix XVIII - Nutritional profile of freeze-dried rotifers

a) Fatty acid composition (% total fatty acids) of freeze-dried rotifers fed with different diets

Structure	FAME	Rotifers fed with b112-PD1	Rotifers fed with bPOME-PD1	Rotifers fed with cPOME-PD1	Rotifers fed with *Nannochloropsis* sp.
Saturated fatty acids					
C 4:0	Butryic	0.00000	0.00000	0.00000	0.00000
C 6:0	Caproic	0.00000	0.00000	0.00000	0.00000
C 8:0	Caprylic	0.37671	0.46110	0.00000	0.65816
C 10:0	Capric	0.31534	0.74928	0.00000	0.56037
C 11:0	Undecanoic	0.12444	0.04014	0.18091	0.00000
C 12:0	Lauric	2.94847	4.03714	0.52046	5.04978
C 13:0	Tridecanoic	0.00000	0.00000	0.00000	0.00000
C 14:0	Myristic	3.07544	4.37085	2.19350	5.00454
C 15:0	Pentadecanoic	0.43867	0.55223	0.38105	0.59744
C 16:0	Palmitic	17.17328	22.03217	16.05774	24.89005
C 17:0	Heptadecanoic	0.59776	0.50863	0.44583	0.88464
C 18:0	Stearic	9.28358	7.48576	6.91844	7.20219
C 20:0	Arachidic	0.70995	0.35882	0.68036	0.28779
C 21:0	Henicosanoic	0.34871	0.40644	9.45236	0.39459
C 22:0	Behenic	1.15219	0.19548	0.13086	0.66467
C 23:0	Tricosanoic	0.00000	0.00000	0.00000	0.00000
C 24:0	Lignoceric	0.00000	0.00000	0.00000	0.00000
Total		36.54454	41.19804	36.96151	46.19422
Unsaturated fatty acids					
C 14:1	Myristoleic	0.46989	0.17979	0.24894	0.28916
C 15:1	Cis-10-Pentadecenoic	0.00000	0.05238	0.19647	0.00000
C 16:1	Palmitoleic	4.36221	4.90956	3.25051	8.18995
C 17:1	Cis-10-Heptadecanoic	0.59692	0.47500	0.28764	0.25758

Appendix XVIIIa, continued

Structure	FAME	Rotifers fed with b112-PD1	Rotifers fed with bPOME-PD1	Rotifers fed with cPOME-PD1	Rotifers fed with *Nannochloropsis* sp.
C 18:1n9c	Oleic	17.52477	25.79256	14.09611	14.75774
C 18:1n9t	Elaidic (Trans)	4.82512	3.75540	3.61619	4.14951
C 20:1n9	Cis-11-Eicosenoic	3.44404	3.36198	5.66783	2.75319
C 22:1n9	Erucic	2.07355	0.45237	1.12443	0.81181
C 24:1	Nervonic	0.00000	1.95442	0.57804	2.40946
Total		33.29650	40.93346	29.06616	33.61840
Polyunsaturated fatty acids					
C 18:2n6c	Linoleic (Cis)	3.75484	4.85306	2.39194	0.80784
C 18:2n6t	Linolelaidic (Trans)	14.29469	1.97830	5.48147	5.14063
C 18:3n3	α-Linolenic	0.44528	0.63980	0.33643	0.00000
C 18:3n6	β-Linolenic	4.81376	4.00093	4.91276	4.45944
C 20:2	Cis-11,14-Eicosadienoic	4.31342	0.40923	4.75270	1.37838
C 20:3n3	Cis-11,14,17-Eicosatrienoic	0.62509	1.00402	0.00000	0.00000
C 20:3n6	Cis-8,11,14-Eicosatrienoic	0.21951	0.52062	9.95085	0.77536
C 20:4n6	Arachidonic (ARA)	0.00000	0.66471	1.87814	1.95348
C 20:5n3	Cis-5,8,11,14,17-eicosapentaenoic (EPA)	0.90226	2.59303	1.57132	4.38366
C 22:2	Cis-13,16 Docosadienoic	0.00000	0.00000	0.00000	0.00000
C 22:6n3	Cis-4,7,10,13,16,19-Docosahexaenoic (DHA)	0.79011	1.20482	2.69672	1.28858
Total		30.15896	17.86852	33.97233	20.18737

b) Amino acid composition (% protein) of freeze-dried rotifers fed with different diets

Amino acid profile	Rotifers fed with b112-PD1	Rotifers fed with bPOME-PD1	Rotifers fed with cPOME-PD1	Rotifers fed with *Nannochloropsis* sp.
Essential amino acids				
Histidine	0.868	0.884	1.109	1.061
Threonine	1.996	2.099	2.434	2.383
Valine	3.885	3.734	4.442	4.508
Methionine	0.946	0.920	0.951	1.026
Lysine	2.404	2.366	3.264	3.538
Isoleucine	2.509	2.399	3.019	3.117
Leucine	3.847	3.643	4.600	4.658
Phenylalanine	2.512	2.404	3.095	3.156
Arginine	2.443	2.441	3.346	3.297
Total	21.410	20.890	26.260	26.744
Non-essential amino acids				
Aspartic acid	3.528	3.443	4.336	4.467
Serine	2.944	2.900	3.820	3.456
Glutamic acid	5.108	4.800	6.043	5.691
Glycine	2.242	2.215	2.839	2.866
Alanine	2.448	2.417	3.060	3.188
Proline	5.890	5.513	5.171	6.479
Tyrosine	2.376	2.296	2.723	2.659
Total	24.536	23.584	27.992	28.806

Chapter 6: Phototrophic bacteria grown in palm oil mill effluent as a feed for the larval marble goby, *Oxyeleotris marmorata* (Bleeker)

Appendix XIX - Summary results of three-way ANOVA and posthoc Tukey HSD test on the survival and growth of larvae directly fed with either bPOME-PD1 or microalgae, in 5 ppt and 10 ppt salinity (Experiment 1)

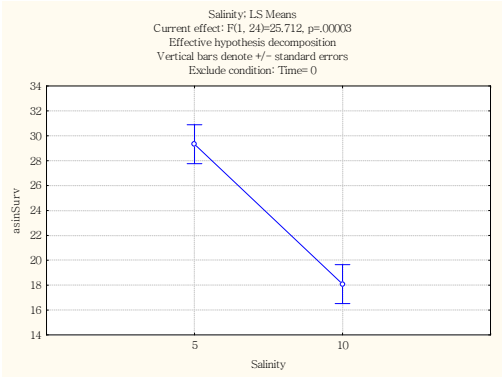
a) Survival

(i) Main effects (shaded area indicates significance at P < 0.02)

Effect	Univariate Tests of Significance for Survival (asin% Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Time= 0				
	SS	Degr. of Freedom	MS	F	p
Intercept	20228.80	1	20228.80	457.8562	0.00000
Salinity	1135.98	1	1135.98	25.7116	0.00003
Feed	8989.89	1	8989.89	203.4762	0.00000
Day of culture	2861.56	2	1430.78	32.3841	0.00000
Salinity*Feed	187.93	1	187.93	4.2535	0.05015
Salinity*Day of culture	1029.70	2	514.85	11.6530	0.00029
Feed*Day of culture	473.06	2	236.53	5.3536	0.01195
Salinity*Feed*Day of culture	1223.44	2	611.72	13.8456	0.00010
Error	1060.36	24	44.18		

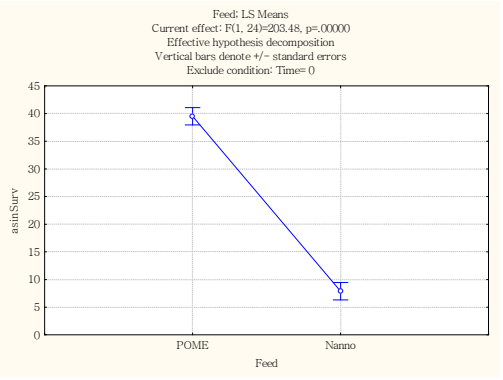
(ii) Salinity

Cell No.	Tukey HSD test; variable asinSurv Homogenous Groups, alpha = .05000 Error: Between MS = 44.182, df = 24.0 Exclude condition: Time= 0			
	Salinity	asinSurv Mean	1	2
2	10	18.08728	****	
1	5	29.32204		****



(iii) Feed

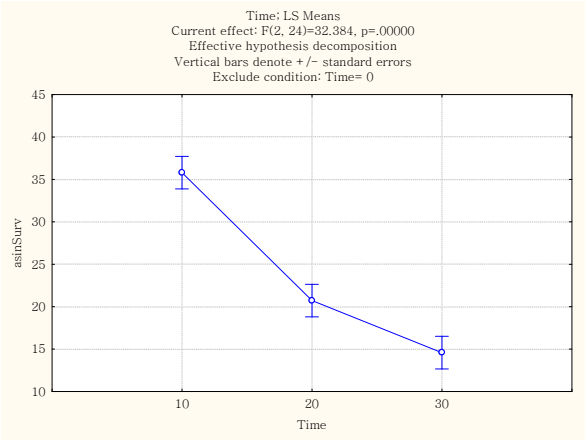
Cell No.	Tukey HSD test; variable asinSurv Homogenous Groups, alpha = .05000 Error: Between MS = 44.182, df = 24.0 Exclude condition: Time= 0			
	Feed	asinSurv Mean	1	2
2	Nanno	7.90215	****	
1	POME	39.50717		****



(iv) Day of culture

Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 44.182, df = 24.000
Exclude condition: Time= 0

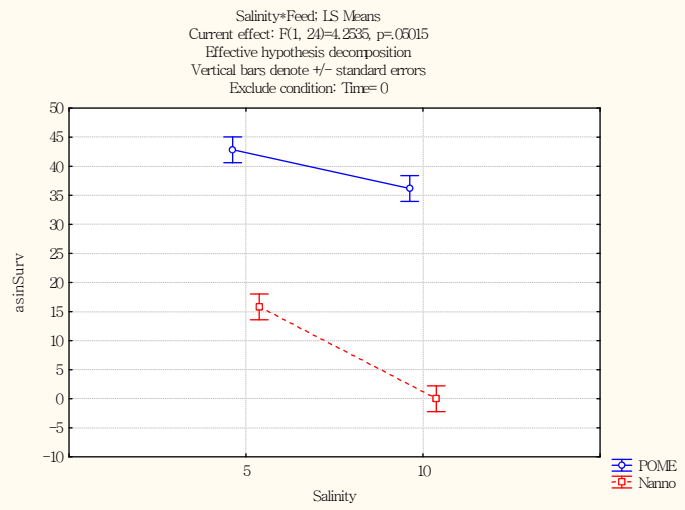
Cell No.	Day of culture	asinSurv Mean	1	2
3	30	14.58529	****	
2	20	20.72384	****	
1	10	35.80486		****



(v) Salinity x Feed interaction

Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 44.182, df = 24.000
Exclude condition: Time= 0

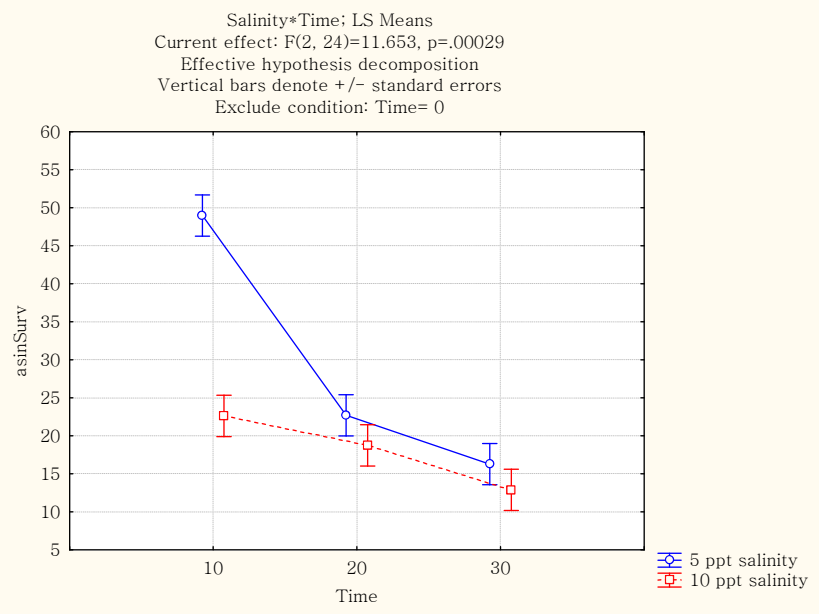
Cell No.	Salinity	Feed	asinSurv Mean	1	2	3
4	10	Nanno	0.00000		****	
2	5	Nanno	15.80431			****
3	10	POME	36.17457	****		
1	5	POME	42.83977	****		



(vi) Salinity x Day of culture interaction

Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 44.182, df = 24.000
Exclude condition: Time= 0

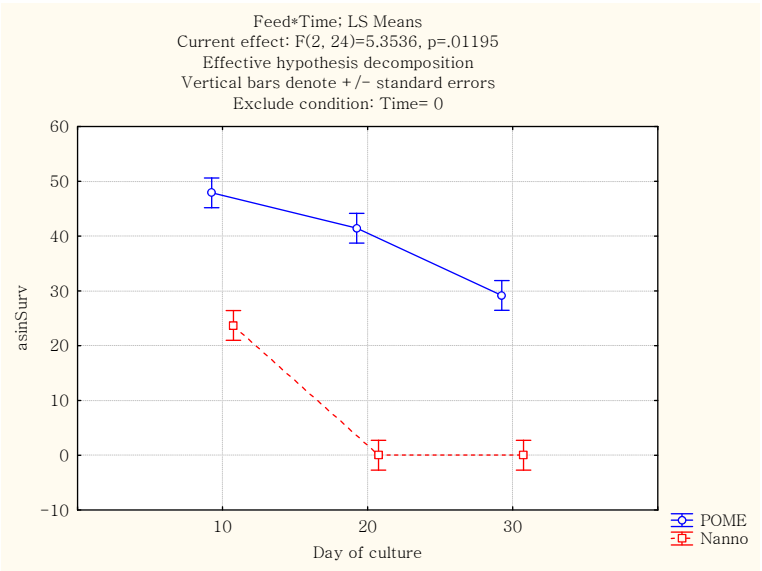
Cell No.	Salinity	Day of culture	asinSurv Mean	1	2
6	10	30	12.89164	****	
3	5	30	16.27894	****	
5	10	20	18.74436	****	
4	10	10	22.62586	****	
2	5	20	22.70332	****	
1	5	10	48.98386		****



(vii) Feed x Day of culture interaction

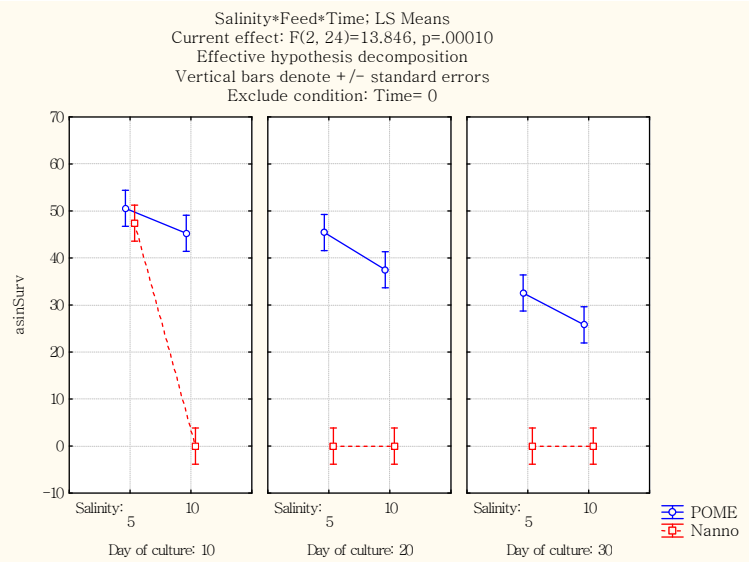
Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 44.182, df = 24.000
Exclude condition: Time= 0

Cell No.	Feed	Day of culture	asinSurv Mean	1	2	3
5	Nanno	20	0.00000	****		
6	Nanno	30	0.00000	****		
4	Nanno	10	23.70646		****	
3	POME	30	29.17057		****	
2	POME	20	41.44767			****
1	POME	10	47.90326			****



(viii) Salinity x Feed x Day of culture interaction

Cell No.	Salinity	Feed	Day of culture	asinSurv Mean	1	2	3
Tukey HSD test; variable asinSurv Homogenous Groups, alpha = .05000 Error: Between MS = 44.182, df = 24.000 Exclude condition: Time= 0							
12	10	Nanno	30	0.00000		****	
11	10	Nanno	20	0.00000		****	
6	5	Nanno	30	0.00000		****	
10	10	Nanno	10	0.00000		****	
5	5	Nanno	20	0.00000		****	
9	10	POME	30	25.78327			****
3	5	POME	30	32.55787	****		****
8	10	POME	20	37.48871	****		****
7	10	POME	10	45.25172	****		****
2	5	POME	20	45.40663	****		
4	5	Nanno	10	47.41292	****		
1	5	POME	10	50.55481	****		



b) Total length

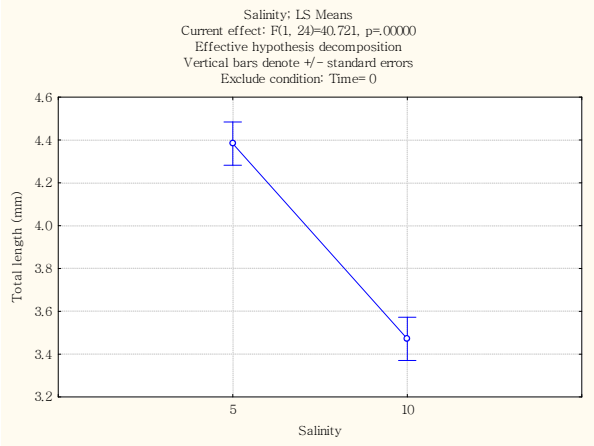
(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for Total length (mm Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Time= 0					
Intercept	555.3092	1	555.3092	3023.020	0.000000
Salinity	7.4802	1	7.4802	40.721	0.000001
Feed	338.0082	1	338.0082	1840.066	0.000000
Day of culture	2.9665	2	1.4832	8.074	0.002082
Salinity*Feed	5.9780	1	5.9780	32.543	0.000007
Salinity*Day of culture	13.8780	2	6.9390	37.775	0.000000
Feed*Day of culture	54.2485	2	27.1242	147.660	0.000000
Salinity*Feed*Day of culture	13.2909	2	6.6455	36.177	0.000000
Error	4.4086	24	0.1837		

(ii) Salinity

Tukey HSD test; variable Total length (mm
Homogenous Groups, alpha = .05000
Error: Between MS = .18369, df = 24.000
Exclude condition: Time= 0

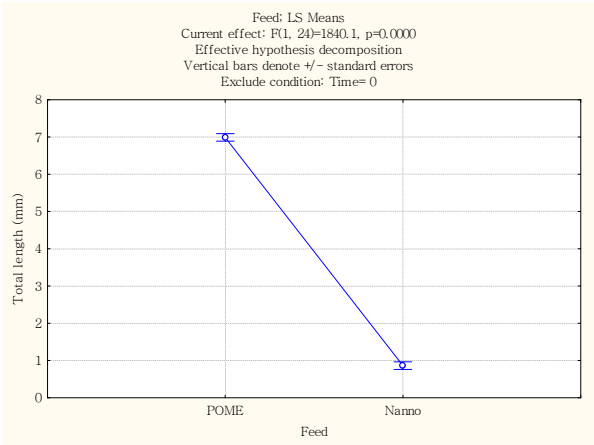
Cell No.	Salinity	Total length (mm) Mean	1	2
2	10	3.471667	****	
1	5	4.383333		****



(iii) Feed

Tukey HSD test; variable Total length (mm
Homogenous Groups, alpha = .05000
Error: Between MS = .18369, df = 24.000
Exclude condition: Time= 0

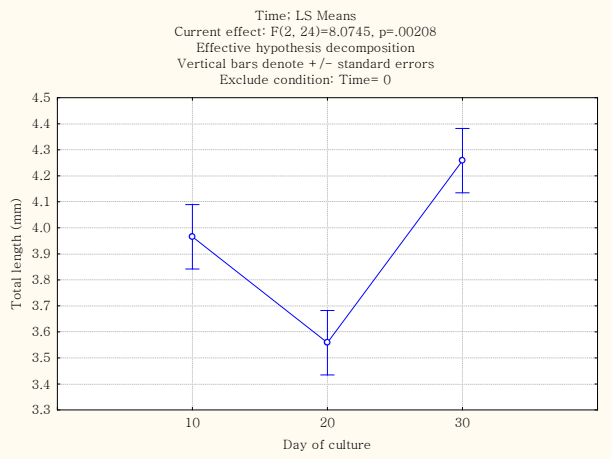
Cell No.	Feed	Total length (mm) Mean	1	2
2	Nanno	0.863333	****	
1	POME	6.991667		****



(iv) Day of culture

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .18369, df = 24.000
Exclude condition: Time= 0

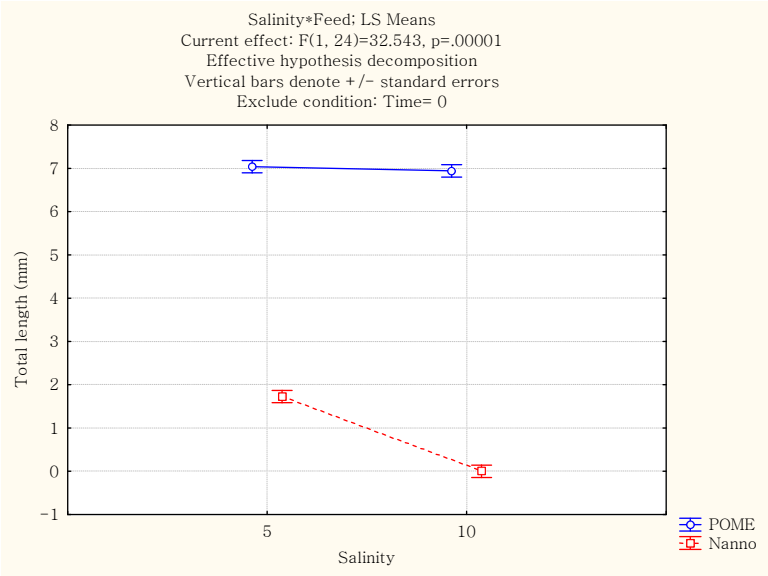
Cell No.	Day of culture	Total length (mm) Mean	1	2
2	20	3.558333	****	
1	10	3.965833	****	****
3	30	4.258333		****



(v) Salinity x Feed interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .18369, df = 24.000
Exclude condition: Time= 0

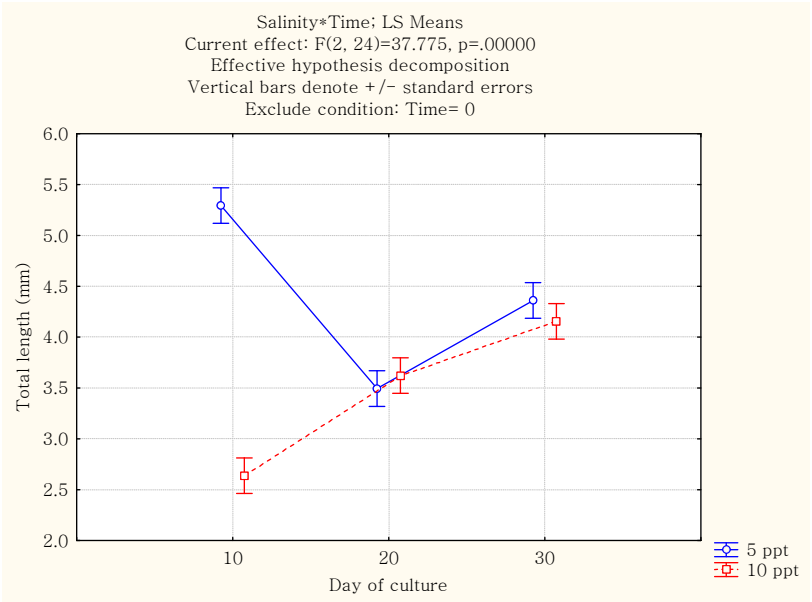
Cell No.	Salinity	Feed	Total length (mm) Mean	1	2	3
4	10	Nanno	0.000000		****	
2	5	Nanno	1.726667			****
3	10	POME	6.943333	****		
1	5	POME	7.040000	****		



(vi) Salinity x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .18369, df = 24.000
Exclude condition: Time= 0

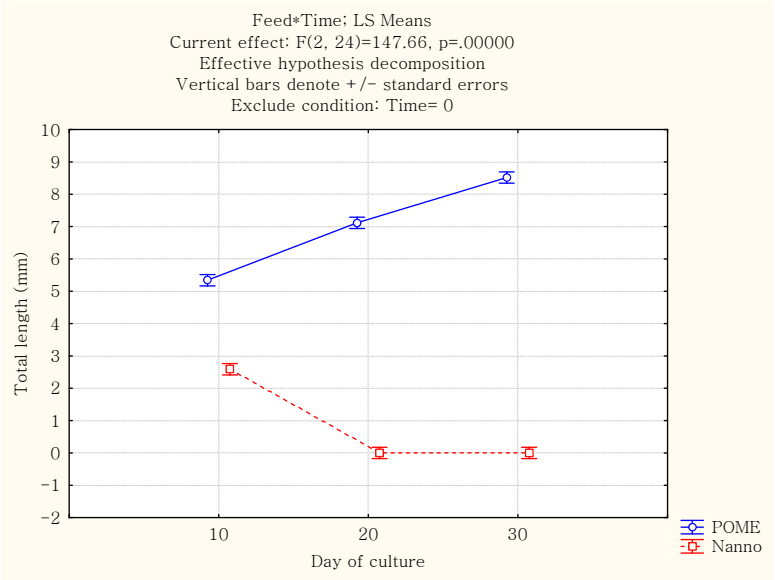
Cell No.	Salinity	Day of culture	Total length (mm) Mean	1	2	3	4
4	10	10	2.637222			****	
2	5	20	3.494444	****			
5	10	20	3.622222	****	****		
6	10	30	4.155556	****	****		
3	5	30	4.361111		****		
1	5	10	5.294444				****



(vii) Feed x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .18369, df = 24.000
Exclude condition: Time= 0

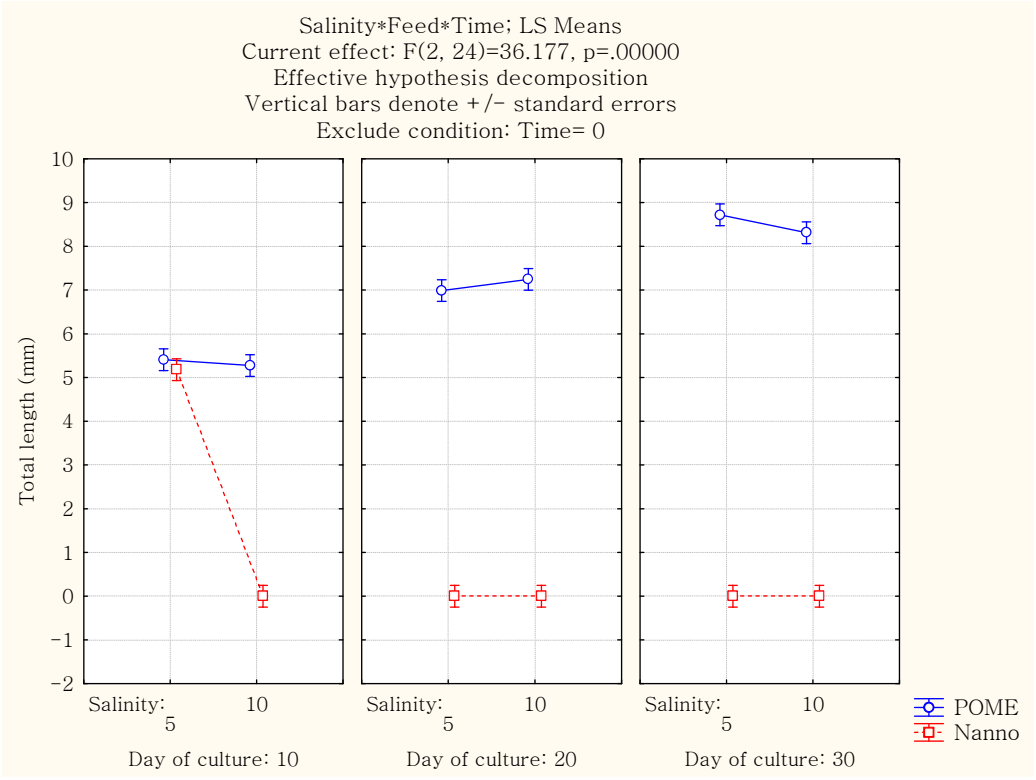
Cell No.	Feed	Day of culture	Total length (mm) Mean	1	2	3	4	5
5	Nanno	20	0.000000	****				
6	Nanno	30	0.000000	****				
4	Nanno	10	2.590000		****			
1	POME	10	5.341667			****		
2	POME	20	7.116667				****	
3	POME	30	8.516667					****



(viii) Salinity x Feed x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .18369, df = 24.000
Exclude condition: Time= 0

Cell No.	Salinity	Feed	Day of culture	Total length (mm) Mean	1	2	3	4	5
11	10	Nanno	20	0.000000	****				
10	10	Nanno	10	0.000000	****				
6	5	Nanno	30	0.000000	****				
5	5	Nanno	20	0.000000	****				
12	10	Nanno	30	0.000000	****				
4	5	Nanno	10	5.180000		****			
7	10	POME	10	5.274444		****			
1	5	POME	10	5.408889		****			
2	5	POME	20	6.988889			****		
8	10	POME	20	7.244444			****	****	
9	10	POME	30	8.311111				****	****
3	5	POME	30	8.722222					****


Salinity*Feed*Time; LS Means
Current effect: F(2, 24)=36.177, p=.00000
Effective hypothesis decomposition
Vertical bars denote +/- standard errors
Exclude condition: Time= 0
Total length (mm)
10
9
8
7
6
5
4
3
2
1
0
-1
-2
Salinity: 5
10
Day of culture: 10
Day of culture: 20
Day of culture: 30
POME
Nanno

Appendix XX –Summary results of t-test on the survival and growth of larvae fed with live feed cultured using bPOME-PD1, in 5 ppt and 10 ppt salinity (Experiment 2)

a) Survival (shaded area indicates significance at P < 0.01)

Variable	T-tests; Grouping: Feed Group 1: 5ppt salinity Group 2: 10ppt salinity Include condition: Time = 30 Mean 5ppt salinity	Mean 10ppt salinity	t-value	df	p	Valid N 5ppt salinity	Valid N 10ppt salinity	Std.Dev. 5ppt salinity	Std.Dev. 10ppt salinity	F-ratio Variances	p Variances
Survival (%)	51.63333	18.36667	4.864417	4	0.008253	3	3	3.980368	11.15631	7.855880	0.225839

b) Total length (shaded area indicates significance at P < 0.01)

Variable	T-tests; Grouping: Feed Group 1: 5ppt salinity Group 2: 10ppt salinity Include condition: Time = 30 Mean 5ppt salinity	Mean 10ppt salinity	t-value	df	p	Valid N 5ppt salinity	Valid N 10ppt salinity	Std.Dev. 5ppt salinity	Std.Dev. 10ppt salinity	F-ratio Variances	p Variances
Total length (mm)	7.677267	8.614567	-6.55097	4	0.002807	3	3	0.175706	0.174761	1.010843	0.994608

Appendix XXI - Summary results of three-way ANOVA and posthoc Tukey HSD test on the effects of feed, salinity and day of culture on larval survival and growth (Experiment 3)

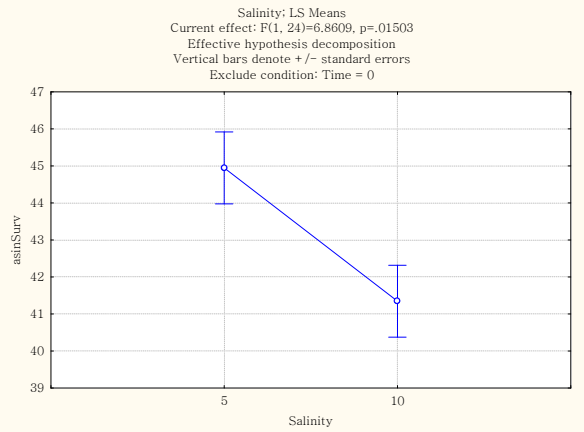
a) Survival

(i) Main effects (shaded area indicates significance at P < 0.02)

Effect	Univariate Tests of Significance for Survival (asin%) Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Time = 0				
	SS	Degr. of Freedom	MS	F	p
Intercept	67020.77	1	67020.77	3942.940	0.000000
Salinity	116.62	1	116.62	6.861	0.015032
Feed	311.33	1	311.33	18.316	0.000259
Day of culture	330.20	2	165.10	9.713	0.000812
Salinity*Feed	42.26	1	42.26	2.486	0.127944
Salinity*Day of culture	21.93	2	10.97	0.645	0.533419
Feed*Day of culture	22.66	2	11.33	0.667	0.522666
Salinity*Feed*Day of culture	1.36	2	0.68	0.040	0.960981
Error	407.94	24	17.00		

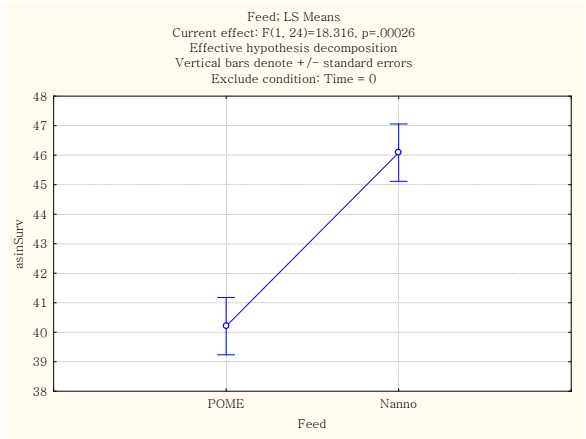
(ii) Salinity

Cell No.	Tukey HSD test; variable asinSurv Homogenous Groups, alpha = .05000 Error: Between MS = 16.998, df = 24.00 Exclude condition: Time = 0			
	Salinity	asinSurv Mean	1	2
2	10	41.34745	****	
1	5	44.94712		****



(iii) Feed

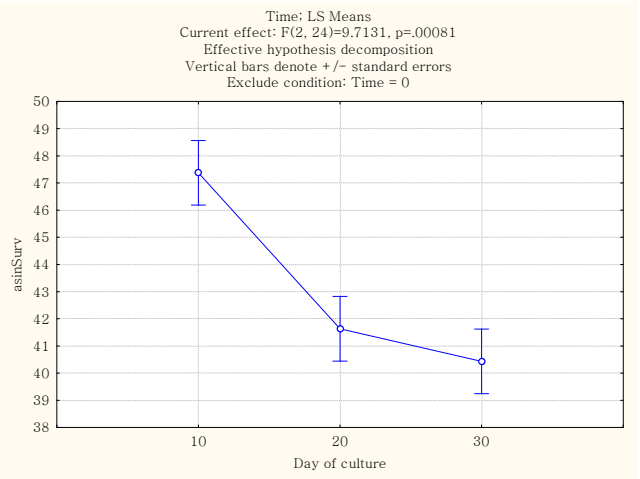
Cell No.	Tukey HSD test; variable asinSurv Homogenous Groups, alpha = .05000 Error: Between MS = 16.998, df = 24.00 Exclude condition: Time = 0			
	Feed	asinSurv Mean	1	2
1	POME	40.20653	****	
2	Nanno	46.08804		****



(iv) Day of culture

Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 16.998, df = 24.00
Exclude condition: Time = 0

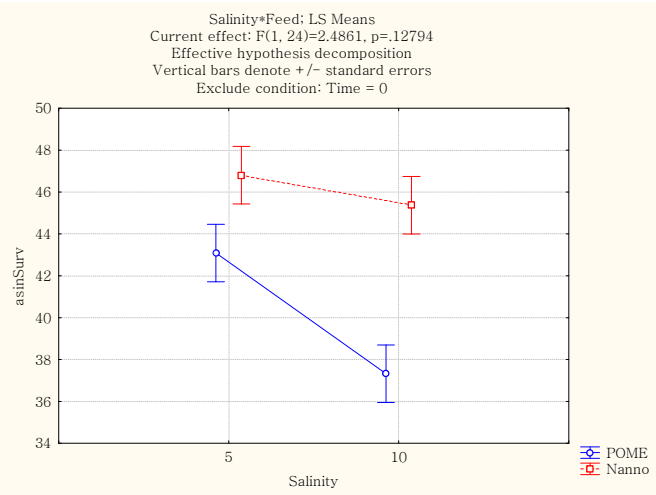
Cell No.	Day of culture	asinSurv Mean	1	2
3	30	40.43397	****	
2	20	41.63396	****	
1	10	47.37393		****



(v) Salinity x Feed interaction

Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 16.998, df = 24.000
Exclude condition: Time = 0

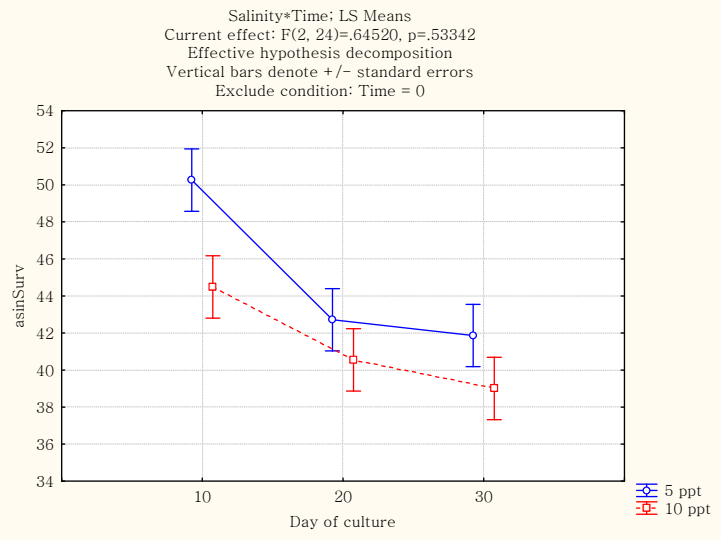
Cell No.	Salinity	Feed	asinSurv Mean	1	2
3	10	POME	37.32325		****
1	5	POME	43.08981	****	
4	10	Nanno	45.37165	****	
2	5	Nanno	46.80443	****	



(vi) Salinity x Day of culture interaction

Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 16.998, df = 24.000
Exclude condition: Time = 0

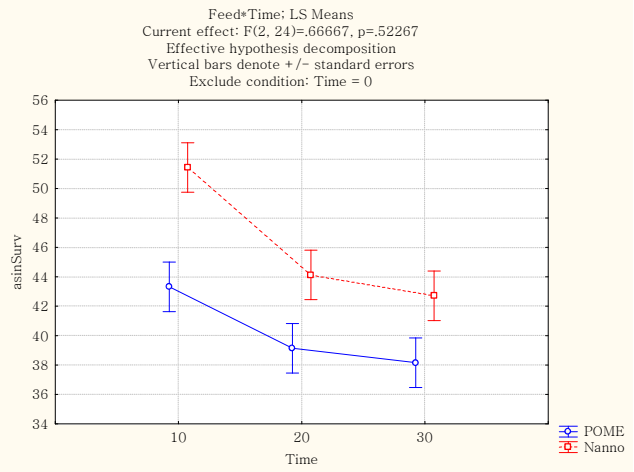
Cell No.	Salinity	Day of culture	asinSurv Mean	1	2
6	10	30	39.00286	****	
5	10	20	40.55084	****	
3	5	30	41.86509	****	
2	5	20	42.71708	****	
4	10	10	44.48866	****	****
1	5	10	50.25920		****



(vii) Feed x Day of culture interaction

Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 16.998, df = 24.000
Exclude condition: Time = 0

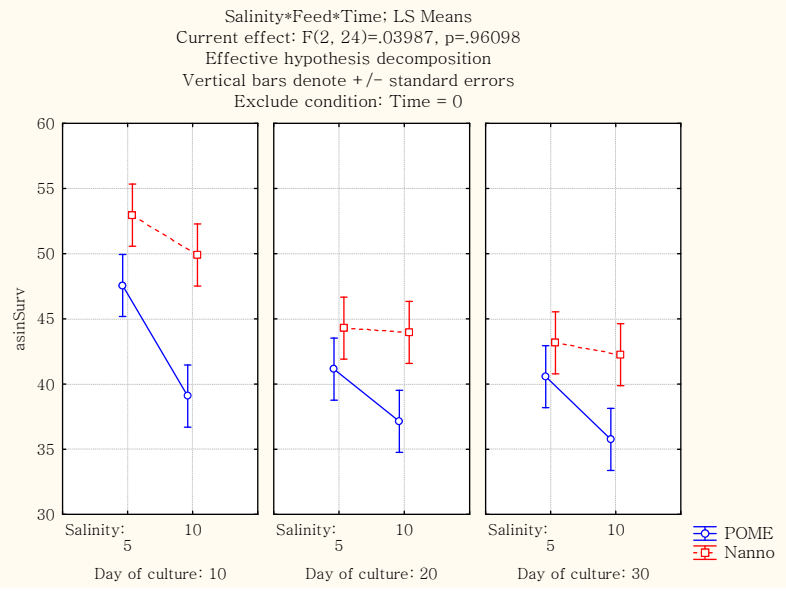
Cell No.	Feed	Day of culture	asinSurv Mean	1	2
3	POME	30	38.16089	****	
2	POME	20	39.14037	****	
6	Nanno	30	42.70705	****	
1	POME	10	43.31832	****	
5	Nanno	20	44.12754	****	****
4	Nanno	10	51.42953		****



(viii) Salinity x Feed x Day of culture interaction

Tukey HSD test; variable asinSurv
Homogenous Groups, alpha = .05000
Error: Between MS = 16.998, df = 24.000
Exclude condition: Time = 0

Cell No.	Salinity	Feed	Day of culture	asinSurv Mean	1	2	3
9	10	POME	30	35.75459	****		
8	10	POME	20	37.13804	****		
7	10	POME	10	39.07712	****	****	
3	5	POME	30	40.56720	****	****	
2	5	POME	20	41.14270	****	****	****
12	10	Nanno	30	42.25113	****	****	****
6	5	Nanno	30	43.16297	****	****	****
11	10	Nanno	20	43.96363	****	****	****
5	5	Nanno	20	44.29145	****	****	****
1	5	POME	10	47.55952	****	****	****
10	10	Nanno	10	49.90019		****	****
4	5	Nanno	10	52.95887			****



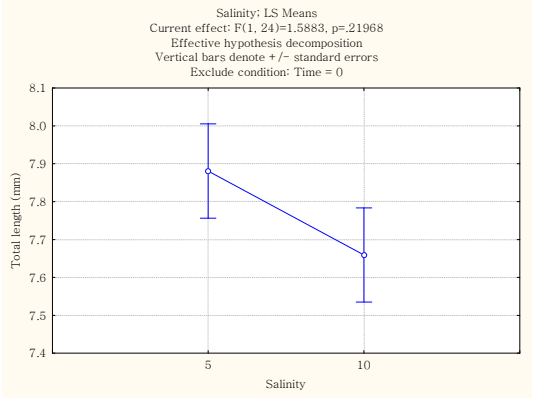
b) Total length

(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	Univariate Tests of Significance for Total length (mr Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Time = 0 SS	Degr. of Freedom	MS	F	p
Intercept	2173.476	1	2173.476	7806.308	0.000000
Salinity	0.442	1	0.442	1.588	0.219685
Feed	2.892	1	2.892	10.387	0.003634
Day of culture	186.417	2	93.209	334.771	0.000000
Salinity*Feed	0.195	1	0.195	0.701	0.410834
Salinity*Day of culture	0.499	2	0.250	0.897	0.421062
Feed*Day of culture	0.165	2	0.082	0.296	0.746403
Salinity*Feed*Day of culture	0.302	2	0.151	0.542	0.588256
Error	6.682	24	0.278		

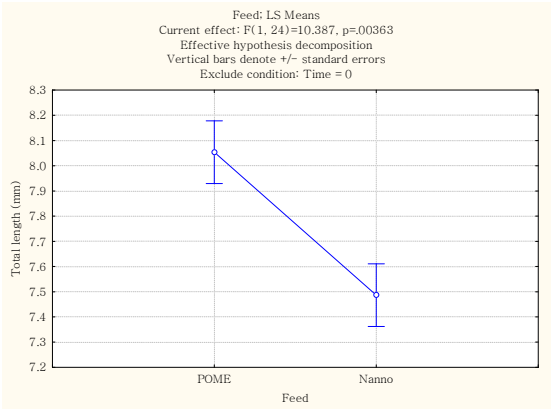
(ii) Salinity

Cell No.	Tukey HSD test; variable Total length (mm Homogenous Groups, alpha = .05000 Error: Between MS = .27843, df = 24.000 Exclude condition: Time = 0 Salinity	Total length (mm) Mean	1
2	10	7.659259	****
1	5	7.880926	****



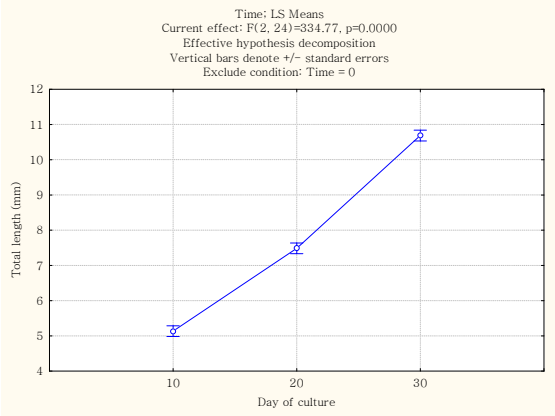
(iii) Feed

Cell No.	Tukey HSD test; variable Total length (mm Homogenous Groups, alpha = .05000 Error: Between MS = .27843, df = 24.000 Exclude condition: Time = 0 Feed	Total length (mm) Mean	1	2
2	Nanno	7.486667	****	
1	POME	8.053519		****



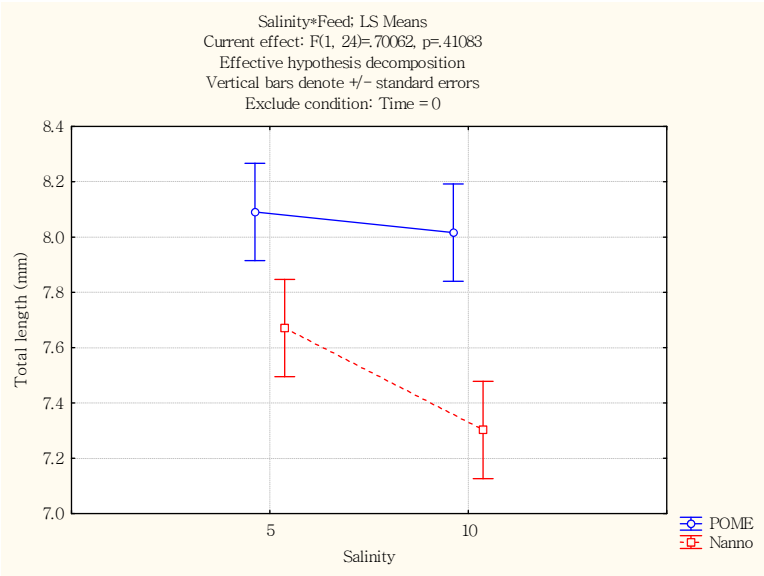
(iv) Day of culture

Cell No.	Day of culture	Total length (mm) Mean	1	2	3
Tukey HSD test; variable Total length (mm) Homogenous Groups, alpha = .05000 Error: Between MS = .27843, df = 24.000 Exclude condition: Time = 0					
1	10	5.13611	****		
2	20	7.48583		****	
3	30	10.68833			****



(v) Salinity x Feed interaction

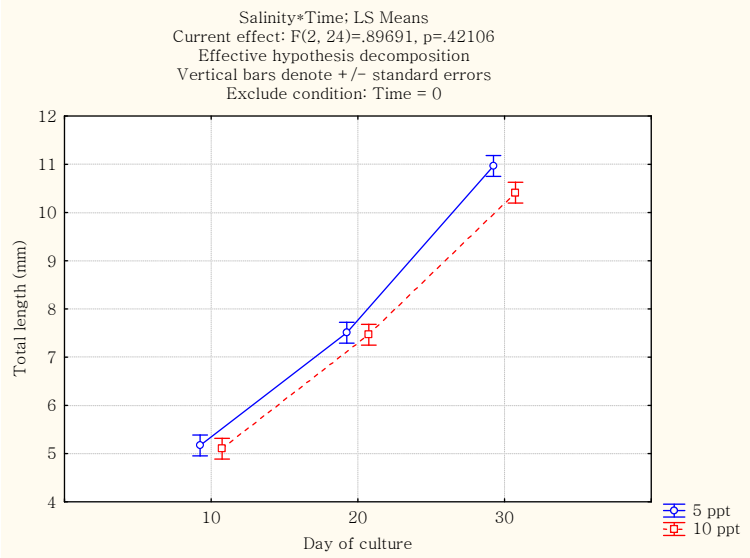
Cell No.	Salinity	Feed	Total length (mm) Mean	1	2
Tukey HSD test; variable Total length (mm) Homogenous Groups, alpha = .05000 Error: Between MS = .27843, df = 24.000 Exclude condition: Time = 0					
4	10	Nanno	7.302222		****
2	5	Nanno	7.671111	****	****
3	10	POME	8.016296	****	
1	5	POME	8.090741	****	



(vi) Salinity x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .27843, df = 24.000
Exclude condition: Time = 0

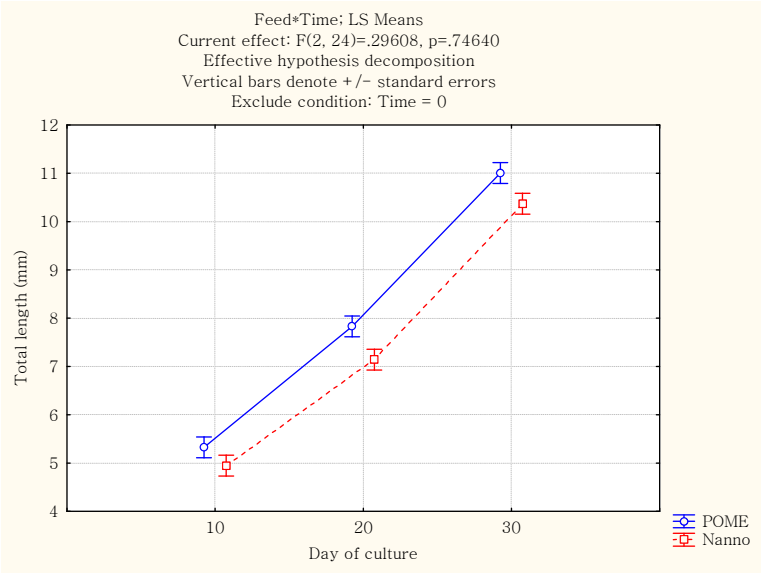
Cell No.	Salinity	Day of culture	Total length (mm) Mean	1	2	3
4	10	10	5.10167	****		
1	5	10	5.17056	****		
5	10	20	7.46500		****	
2	5	20	7.50667		****	
6	10	30	10.41111			****
3	5	30	10.96556			****



(vii) Feed x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .27843, df = 24.000
Exclude condition: Time = 0

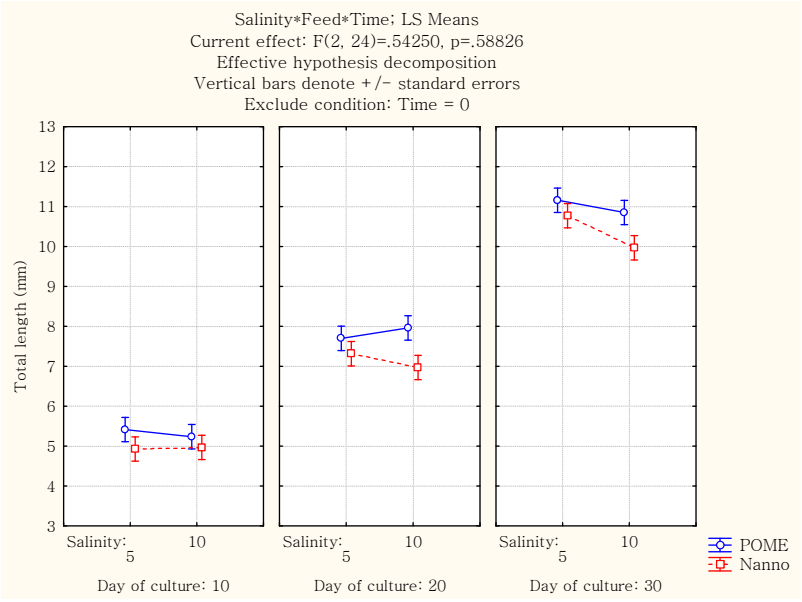
Cell No.	Feed	Time	Total length (mm) Mean	1	2	3
4	Nanno	10	4.94722	****		
1	POME	10	5.32500	****		
5	Nanno	20	7.14222		****	
2	POME	20	7.82944		****	
6	Nanno	30	10.37056			****
3	POME	30	11.00611			****



(viii) Salinity x Feed x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .27843, df = 24.000
Exclude condition: Time = 0

Cell No.	Salinity	Feed	Day of culture	Total length (mm) Mean	1	2	3
4	5	Nanno	10	4.92667	****		
10	10	Nanno	10	4.96778	****		
7	10	POME	10	5.23556	****		
1	5	POME	10	5.41444	****		
11	10	Nanno	20	6.97000		****	
5	5	Nanno	20	7.31444		****	
2	5	POME	20	7.69889		****	
8	10	POME	20	7.96000		****	
12	10	Nanno	30	9.96889			****
6	5	Nanno	30	10.77222			****
9	10	POME	30	10.85333			****
3	5	POME	30	11.15889			****



Appendix XXII - Fatty acid and amino acid profiles of freeze-dried marble goby larvae

a) Fatty acid composition (% total fatty acids) of freeze-dried fish larvae

Structure	FAME	Larvae given bPOME-PD1	Larvae given *Nannochloropsis* sp.	Larvae given rotifers fed with bPOME-PD1	Larvae given rotifers fed with cPOME-PD1	Larvae given rotifers fed with *Nannochloropsis* sp.
Saturated fatty acids						
C 4:0	Butryic	0.00000	0.00000	0.00000	0.00000	0.00000
C 6:0	Caproic	0.00000	0.00000	0.00000	0.00000	0.16229
C 8:0	Caprylic	0.00000	0.00000	0.17619	0.00000	1.33852
C 10:0	Capric	0.02230	0.00000	0.15191	0.00000	1.22055
C 11:0	Undecanoic	0.02774	0.03354	0.03147	0.00000	0.08743
C 12:0	Lauric	0.16097	0.18785	1.95391	0.30960	12.89653
C 13:0	Tridecanoic	0.00000	0.00000	0.00000	0.00000	0.00000
C 14:0	Myristic	1.16467	1.15839	1.95886	0.83724	7.78108
C 15:0	Pentadecanoic	0.76900	0.77198	0.59273	0.50663	0.35236
C 16:0	Palmitic	28.61199	28.04765	22.74312	20.10674	22.16925
C 17:0	Heptadecanoic	2.19615	2.26608	1.26223	1.33743	1.05592
C 18:0	Stearic	22.01126	20.90668	19.65829	18.24971	19.92809
C 20:0	Arachidic	1.09592	0.95640	0.82824	0.86104	0.88622
C 21:0	Henicosanoic	0.61360	0.60860	0.05640	0.13402	0.00000
C 22:0	Behenic	0.21910	0.21261	0.22200	0.40692	0.44295
C 23:0	Tricosanoic	0.11679	0.12120	0.09001	0.00000	0.00000
C 24:0	Lignoceric	0.11894	0.14095	0.21657	0.33967	0.42387
Total		57.12843	55.41193	49.94193	43.089	68.74506
Monounsaturated fatty acids						
C 14:1	Myristoleic	0.02905	0.03985	0.31875	0.47591	0.18998
C 15:1	Cis-10-Pentadecenoic	0.16275	0.15670	0.18757	0.27180	0.11828
C 16:1	Palmitoleic	1.57785	1.61956	3.13364	2.84159	2.42867

Appendix XXIIa, continued

Structure	FAME	Larvae given bPOME-PD1	Larvae given *Nannochloropsis* sp.	Larvae given rotifers fed with bPOME-PD1	Larvae given rotifers fed with cPOME-PD1	Larvae given rotifers fed with *Nannochloropsis* sp.
C 17:1	Cis-10-Heptadecanoic	0.77387	0.49183	0.32099	0.55754	0.33811
C 18:1n9c	Oleic	15.33328	15.28395	17.19273	18.59678	17.06961
C 18:1n9t	Elaidic (Trans)	0.37830	0.53264	2.00121	2.03230	1.07980
C 20:1n9	Cis-11-Eicosenoic	0.91112	0.80064	1.42619	3.73869	0.84592
C 22:1n9	Erucic	0.05038	0.04757	0.16699	0.21024	0.24318
C 24:1	Nervonic	0.93095	0.00000	0.72046	4.12227	0.63986
Total		20.14755	18.97274	25.46853	32.84712	22.95341
Polyunsaturated fatty acids						
C 18:2n6c	Linoleic (Cis)	6.13296	6.31143	4.84965	1.09140	2.13084
C 18:2n6t	Linolelaidic (Trans)	3.27394	3.22502	3.40868	7.39195	4.48309
C 18:3n3	α-Linolenic	0.54105	0.59767	0.34920	0.35701	0.00000
C 18:3n6	β-Linolenic	0.65727	0.63967	0.26991	5.70431	0.33175
C 20:2	Cis-11,14-Eicosadienoic	0.09671	0.09813	0.75981	0.33016	0.40707
C 20:3n3	Cis-11,14,17-Eicosatrienoic	0.11377	0.13304	3.69837	0.00000	0.00000
C 20:3n6	Cis-8,11,14-Eicosatrienoic	0.86153	0.90100	0.69849	0.70542	0.00000
C 20:4n6	Arachidonic (ARA)	3.59712	4.11140	0.09517	2.30386	0.43506
C 20:5n3	Cis-5,8,11,14,17-eicosapentaenoic (EPA)	0.56692	0.78760	2.11335	1.22607	0.14290
C 22:2	Cis-13,16 Docosadienoic	0.00000	0.00000	0.00000	0.00000	0.00000
C 22:6n3	Cis-4,7,10,13,16,19-Docosahexaenoic (DHA)	6.88275	8.81036	8.34694	4.95369	0.37081
Total		22.72402	25.61532	24.58957	24.06387	8.30152

b) Amino acid composition (% protein) of freeze-dried fish larvae

Amino acid profile	Larvae given bPOME-PD1	Larvae given *Nannochloropsis* sp.	Larvae given rotifers fed with bPOME-PD1	Larvae given rotifers fed with cPOME-PD1	Larvae given rotifers fed with *Nannochloropsis* sp.
Essential amino acids					
Histidine	0.962	1.205	1.019	0.750	0.690
Threonine	2.027	2.319	2.293	1.508	1.461
Valine	2.761	2.810	2.844	1.863	1.866
Methionine	1.291	1.485	1.623	1.068	0.848
Lysine	2.892	3.922	4.527	2.235	2.046
Isoleucine	2.205	2.476	2.526	1.587	1.534
Leucine	3.492	3.962	4.038	2.478	2.433
Phenylalanine	2.079	2.231	2.032	1.472	1.520
Arginine	2.602	2.871	2.820	1.864	1.881
Total	20.311	23.281	23.722	14.825	14.279

Appendix XXIIb, continued

Amino acid profile	Larvae given bPOME-PD1	Larvae given *Nannochloropsis* sp.	Larvae given rotifers fed with bPOME-PD1	Larvae given rotifers fed with cPOME-PD1	Larvae given rotifers fed with *Nannochloropsis* sp.
Non-essential amino acids					
Aspartic acid	3.919	4.280	4.243	3.096	2.737
Serine	2.257	2.305	2.096	1.535	1.546
Glutamic acid	5.298	5.915	6.059	4.019	3.590
Glycine	3.584	3.433	4.193	2.803	2.270
Alanine	2.763	2.888	2.899	2.078	1.940
Proline	2.777	2.628	2.518	1.717	1.834
Tyrosine	1.805	1.944	1.621	1.220	1.205
Total	22.403	23.393	23.629	16.468	15.122

Chapter 7: Improving survival and mass culture of marble goby *Oxyeleotris marmorata* (Bleeker)

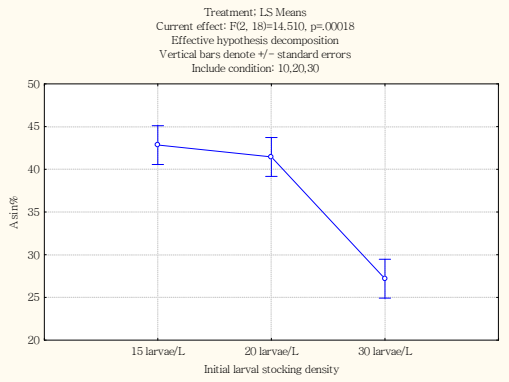
Appendix XXIII - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effect of fish stocking density on survival and growth of fish larvae given live feed fed with bPOME-PD1 (Experiment 1)

a) Survival

(i) Main effects (shaded area indicates significance at P < 0.01)

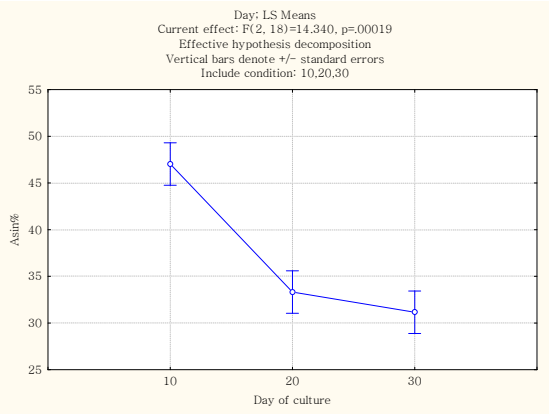
Effect	Univariate Tests of Significance for Survival (Asin% Sigma-restricted parameterization Effective hypothesis decomposition Include condition: 10,20,30				
	SS	Degr. of Freedom	MS	F	p
Intercept	37299.91	1	37299.91	801.5784	0.000000
Initial larval stocking density	1350.37	2	675.18	14.5098	0.000177
Day of culture	1334.59	2	667.30	14.3402	0.000188
Initial larval stocking density*Day of culture	224.66	4	56.17	1.2070	0.342104
Error	837.60	18	46.53		

Cell No.	Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 46.533, df = 18.000 Include condition: 10,20,30			
	Initial larval stocking density	Asin% Mean	1	2
3	30 larvae/L	27.19928		****
2	20 larvae/L	41.45613	****	
1	15 larvae/L	42.84931	****	



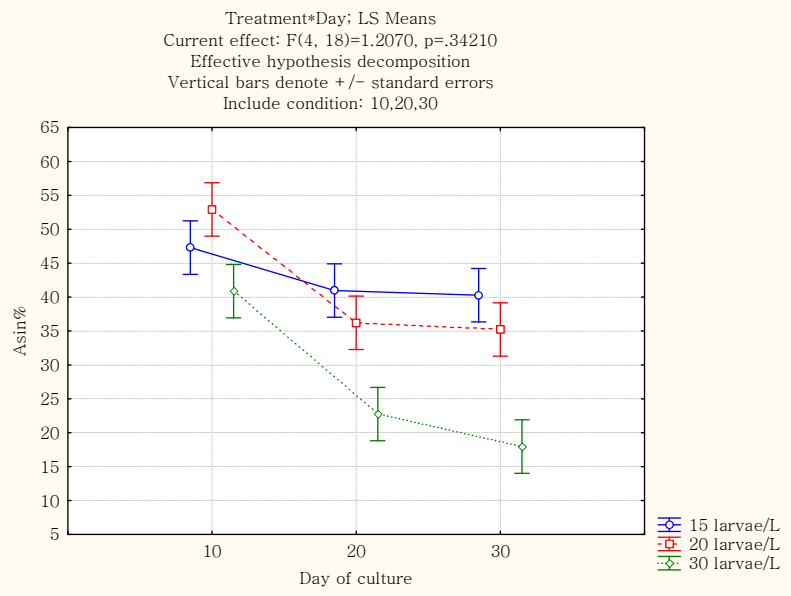
(iii) Day of culture

Cell No.	Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 46.533, df = 18.00(Include condition: 10,20,30			
	Day of culture	Asin% Mean	1	2
3	30	31.15879	****	
2	20	33.31303	****	
1	10	47.03291		****



(iv) Initial larval stocking density x Day of culture interaction

Cell No.	Initial larval stocking density	Day	Asin% Mean	1	2	3
	Tukey HSD test; variable Asin% Homogenous Groups, alpha = .05000 Error: Between MS = 46.533, df = 18.000 Include condition: 10,20,30					
9	30 larvae/L	30	17.96763			****
8	30 larvae/L	20	22.75885		****	****
6	20 larvae/L	30	35.22622	****	****	****
5	20 larvae/L	20	36.21165	****	****	****
3	15 larvae/L	30	40.28252	****	****	
7	30 larvae/L	10	40.87135	****	****	
2	15 larvae/L	20	40.96857	****	****	
1	15 larvae/L	10	47.29685	****		
4	20 larvae/L	10	52.93053	****		



b) Total length

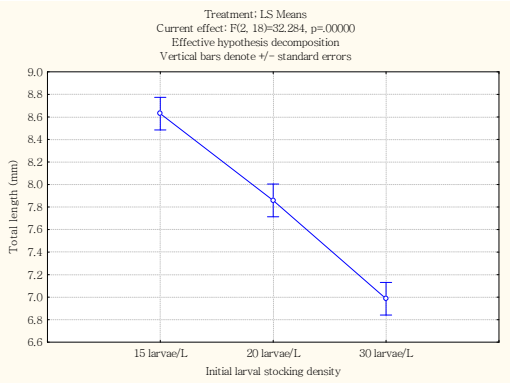
(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	SS	Degr. of Freedom	MS	F	p
	Univariate Tests of Significance for Total length (mm Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1653.149	1	1653.149	8763.954	0.000000
Initial larval stocking density	12.179	2	6.090	32.284	0.000001
Day of culture	82.676	2	41.338	219.149	0.000000
Initial larval stocking density*Day of culture	3.722	4	0.931	4.933	0.007301
Error	3.395	18	0.189		

(ii) Initial larval stocking density

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .18863, df = 18.000

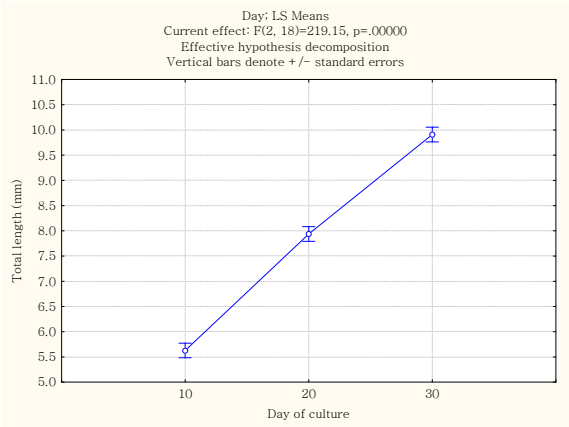
Cell No.	Initial larval stocking density	Total length (mm) Mean	1	2	3
3	30 larvae/L	6.985556	****		
2	20 larvae/L	7.859259		****	
1	15 larvae/L	8.629630			****



(iii) Day of culture

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .18863, df = 18.000

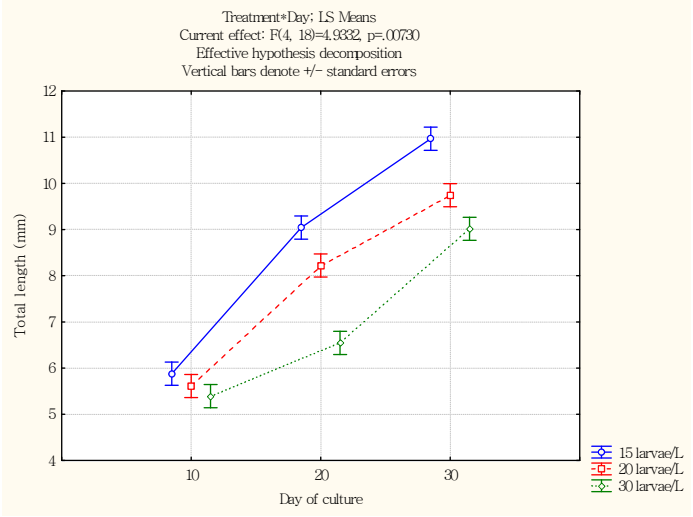
Cell No.	Day of culture	Total length (mm) Mean	1	2	3
1	10	5.627407	****		
2	20	7.937778		****	
3	30	9.909259			****



(iv) Initial larval stocking density x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .18863, df = 18.000

Cell No.	Initial larval stocking density	Day of culture	Total length (mm) Mean	1	2	3	4
7	30 larvae/L	10	5.39333	****			
4	20 larvae/L	10	5.61111	****			
1	15 larvae/L	10	5.87778	****			
8	30 larvae/L	20	6.54667	****			
5	20 larvae/L	20	8.22222		****		
9	30 larvae/L	30	9.01667		****	****	
2	15 larvae/L	20	9.04444		****	****	
6	20 larvae/L	30	9.74444			****	****
3	15 larvae/L	30	10.96667				****



Appendix XXIV - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effect of type of feed (live feed fed with cPOME-PD1 versus live feed fed with POME) on survival and growth of fish larvae (Experiment 2)

a) Survival

(i) Main effects (shaded area indicates significance at P < 0.05)

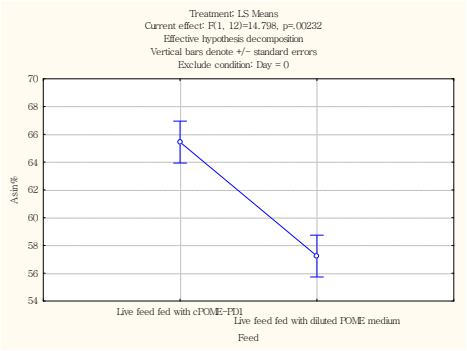
Univariate Tests of Significance for Survival (Asin%)
Sigma-restricted parameterization
Effective hypothesis decomposition
Exclude condition: Day = 0

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	67737.51	1	67737.51	3306.640	0.000000
Feed	303.14	1	303.14	14.798	0.002324
Day of culture	1748.78	2	874.39	42.684	0.000004
Feed*Day of culture	161.48	2	80.74	3.941	0.048333
Error	245.82	12	20.49		

(ii) Feed

Tukey HSD test; variable Asin%
Homogenous Groups, alpha = .05000
Error: Between MS = 20.485, df = 12.000
Exclude condition: Day = 0

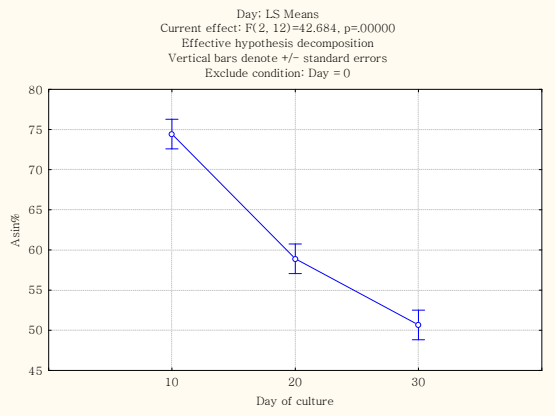
Cell No.	Feed	Asin% Mean	1	2
2	Live feed fed with diluted POME mec	57.24111	****	
1	Live feed fed with cPOME-P	65.44866		****



(iii) Day of culture

Tukey HSD test; variable Asin%
Homogenous Groups, alpha = .05000
Error: Between MS = 20.485, df = 12.000
Exclude condition: Day = 0

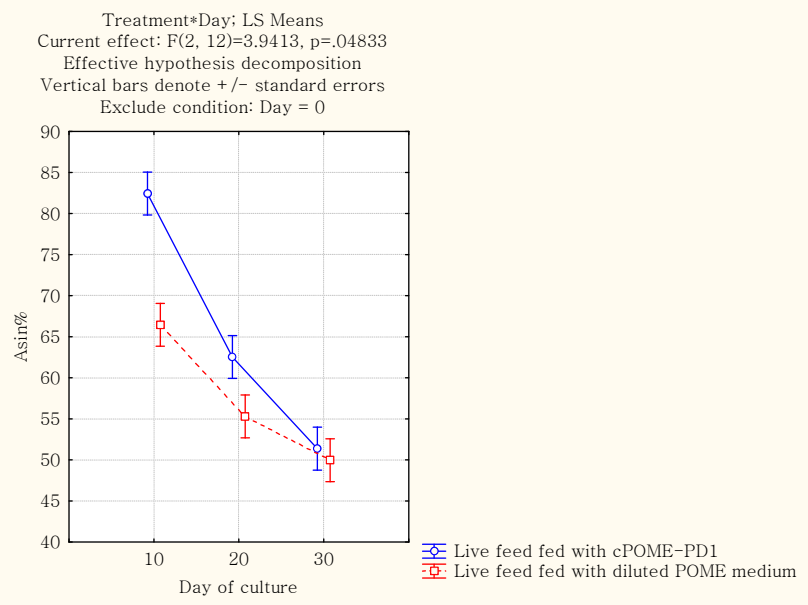
Cell No.	Day of culture	Asin% Mean	1	2	3
3	30	50.67191	****		
2	20	58.91621		****	
1	10	74.44654			****



(iv) Feed x Day of culture interaction

Tukey HSD test; variable Asin%
Homogenous Groups, alpha = .05000
Error: Between MS = 20.485, df = 12.000
Exclude condition: Day = 0

Cell No.	Feed	Day of culture	Asin% Mean	1	2	3	4
6	Live feed with diluted POME mediu	30	49.96888	****			
3	Live feed fed with cPOME-PD	30	51.37494	****	****		
5	Live feed fed with diluted POME mediu	20	55.29900	****	****	****	
2	Live feed fed with cPOME-PD	20	62.53342		****	****	
4	Live feed fed with diluted POME mediu	10	66.45545			****	
1	Live feed fed with cPOME-PD	10	82.43762				****



b) Total length

(i) Main effects (shaded area indicates significance at P < 0.01)

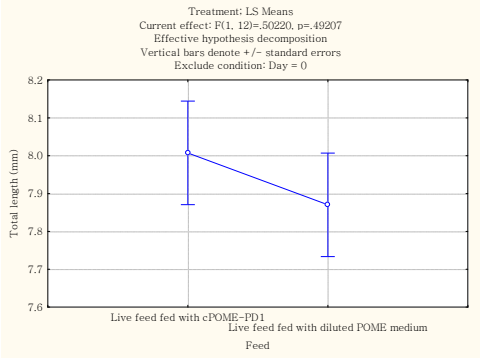
Univariate Tests of Significance for Total length (mm
Sigma-restricted parameterization
Effective hypothesis decomposition
Exclude condition: Day = 0

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1134.467	1	1134.467	6741.882	0.000000
Feed	0.085	1	0.085	0.502	0.492073
Day of culture	62.831	2	31.416	186.696	0.000000
Feed*Day of culture	0.252	2	0.126	0.750	0.493351
Error	2.019	12	0.168		

(ii) Feed

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .16827, df = 12.000
Exclude condition: Day = 0

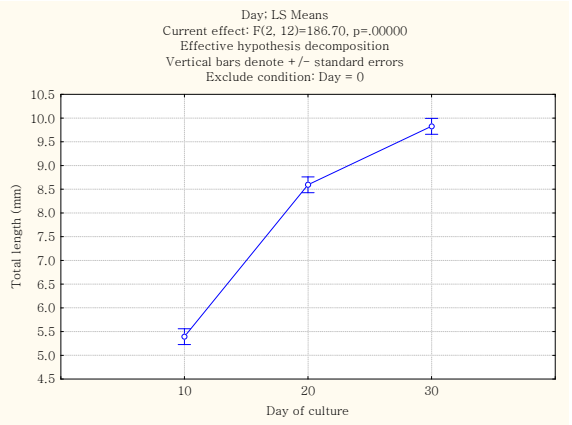
Cell No.	Feed	Total length (mm) Mean	1
2	Live feed with diluted POME med	7.870370	****
1	Live feed fed with cPOME-P	8.007407	****



(iii) Day of culture

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .16827, df = 12.000
Exclude condition: Day = 0

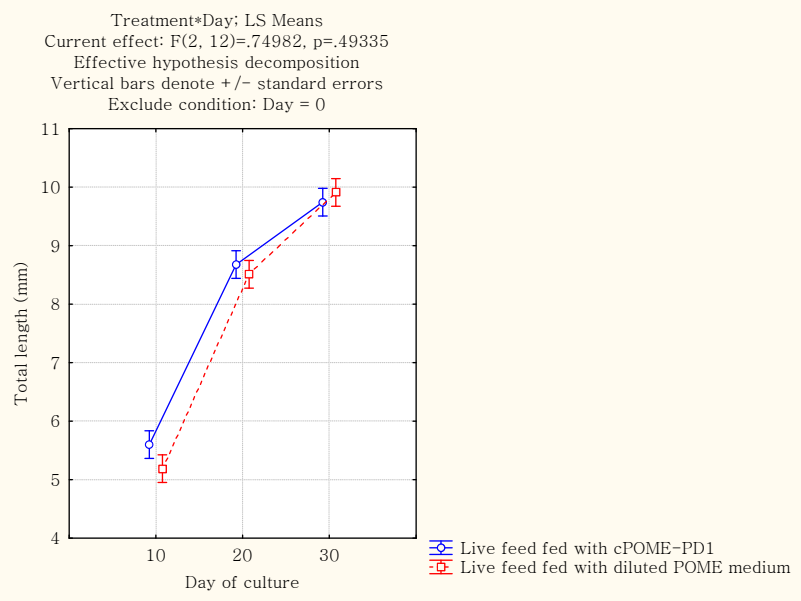
Cell No.	Day of culture	Total length (mm) Mean	1	2	3
1	10	5.394444	****		
2	20	8.594444		****	
3	30	9.827778			****



(iv) Feed x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .16827, df = 12.000
Exclude condition: Day = 0

Cell No.	Feed	Day of culture	Total length (mm) Mean	1	2	3	4
4	Live feed fed with diluted POME mec	10	5.188889	****			
1	Live feed fed with cPOME-P	10	5.600000	****			
5	Live feed fed with diluted POME mec	20	8.511111		****		
2	Live feed fed with cPOME-P	20	8.677778		****	****	
3	Live feed fed with cPOME-P	30	9.744444			****	****
6	Live feed fed with diluted POME mec	30	9.911111				****



Appendix XXV - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effect of tank colour on survival and growth of fish larvae (Experiment 3)

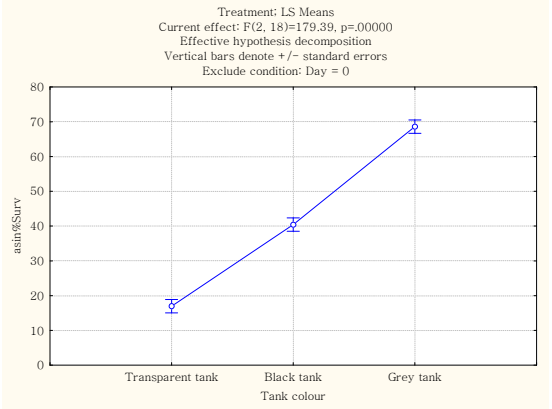
a) Survival

(i) Main effects (shaded area indicates significance at P < 0.01)

Univariate Tests of Significance for Survival (Asin%) Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Day = 0					
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	47646.00	1	47646.00	1420.363	0.000000
Tank colour	12035.44	2	6017.72	179.393	0.000000
Day of culture	2992.49	2	1496.24	44.604	0.000000
Tank colour*Day of culture	933.85	4	233.46	6.960	0.001435
Error	603.81	18	33.54		

(ii) Tank colour

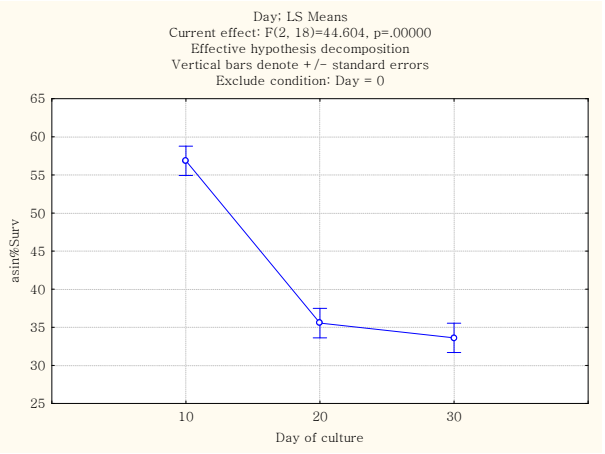
Tukey HSD test; variable asin%Surv Homogenous Groups, alpha = .05000 Error: Between MS = 33.545, df = 18.000 Exclude condition: Day = 0					
Cell No.	Tank colour	asin%Surv Mean	1	2	3
1	Transparent tank	16.96901	****		
2	Black tank	40.44105		****	
3	Grey tank	68.61374			****



(iii) Day of culture

Tukey HSD test; variable asin%Surv
Homogenous Groups, alpha = .05000
Error: Between MS = 33.545, df = 18.00
Exclude condition: Day = 0

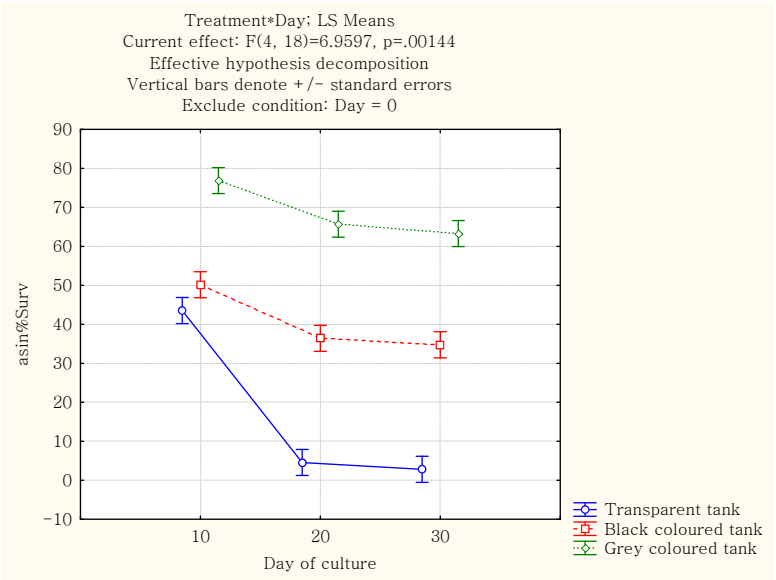
Cell No.	Day of culture	asin%Surv Mean	1	2
3	30	33.61504	****	
2	20	35.55455	****	
1	10	56.85421		****



(iv) Tank colour x Day of culture interaction

Tukey HSD test; variable asin%Surv
Homogenous Groups, alpha = .05000
Error: Between MS = 33.545, df = 18.000
Exclude condition: Day = 0

Cell No.	Tank colour	Day of culture	asin%Surv Mean	1	2	3	4
3	Transparent tank	30	2.79954				****
2	Transparent tank	20	4.56352				****
6	Black tank	30	34.75225	****			
5	Black tank	20	36.41494	****			
1	Transparent tank	10	43.54398	****			
4	Black tank	10	50.15596	****	****		
9	Grey tank	30	63.29333		****	****	
8	Grey tank	20	65.68519		****	****	
7	Grey tank	10	76.86270			****	



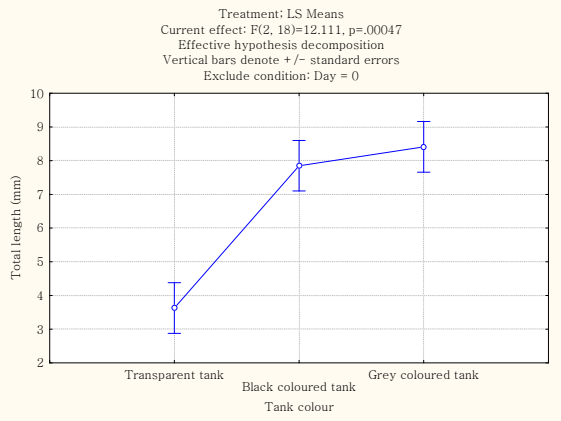
b) Total length

(i) Main effects (shaded area indicates significance at P < 0.02)

Effect	Univariate Tests of Significance for Total length (m Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Day = 0				
	SS	Degr. of Freedom	MS	F	p
Intercept	1187.323	1	1187.323	233.7090	0.000000
Tank colour	123.059	2	61.529	12.1113	0.000465
Day of culture	58.062	2	29.031	5.7144	0.011981
Tank colour*Day of culture	54.127	4	13.532	2.6635	0.066205
Error	91.446	18	5.080		

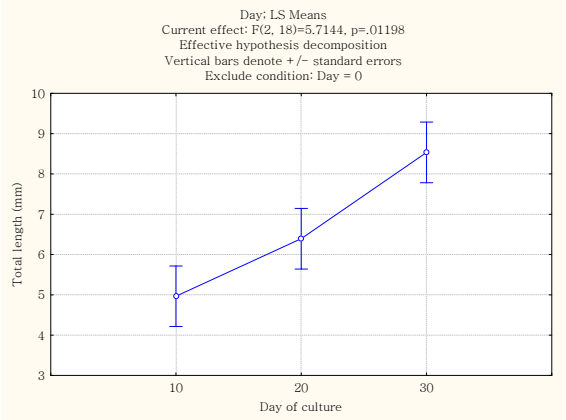
(ii) Tank colour

Cell No.	Tukey HSD test; variable Total length (mm) Homogenous Groups, alpha = .05000 Error: Between MS = 5.0803, df = 18.000 Exclude condition: Day = 0			
	Tank colour	Total length (mm) Mean	1	2
1	Transparent tank	3.629630		****
2	Black tank	7.851481	****	
3	Grey tank	8.412963	****	



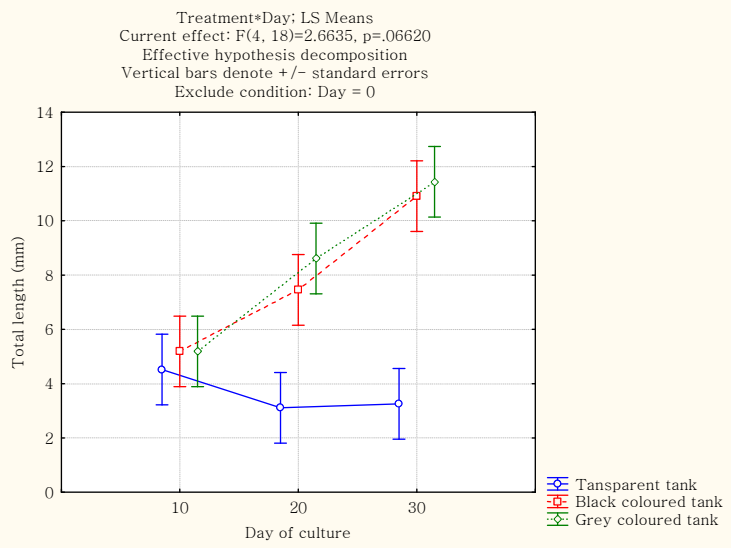
(iii) Day of culture

Cell No.	Tukey HSD test; variable Total length (mm) Homogenous Groups, alpha = .05000 Error: Between MS = 5.0803, df = 18.000 Exclude condition: Day = 0			
	Day of culture	Total length (mm) Mean	1	2
1	10	4.966667	****	
2	20	6.392593	****	****
3	30	8.534815		****



(iv) Tank colour x Day of culture interaction

Cell No.	Tank colour	Day of culture	Total length (mm) Mean	1	2	3
Tukey HSD test; variable Total length (mm) Homogenous Groups, alpha = .05000 Error: Between MS = 5.0803, df = 18.000 Exclude condition: Day = 0						
2	Transparent tank	20	3.11111	****		
3	Transparent tank	30	3.25556	****		
1	Transparent tank	10	4.52222	****	****	
7	Grey tank	10	5.18889	****	****	****
4	Black tank	10	5.18889	****	****	****
5	Black tank	20	7.45556	****	****	****
8	Grey tank	20	8.61111	****	****	****
6	Black tank	30	10.91000		****	****
9	Grey tank	30	11.43889			****



Appendix XXVI - Summary results of two-way ANOVA and posthoc Tukey HSD test on the effect of type of feed (live feed fed with cPOME-PD1 versus live feed fed with bPOME-PD1) on survival and growth of fish larvae (Experiment 4)

a) Survival

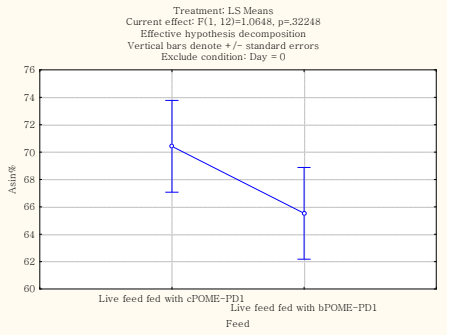
(i) Main effects (shaded area indicates significance at P < 0.05)

Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for Survival (Asin%) Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Day = 0					
Intercept	83172.62	1	83172.62	821.8911	0.000000
Feed	107.75	1	107.75	1.0648	0.322478
Day of culture	818.27	2	409.13	4.0430	0.045472
Feed*Day of culture	5.33	2	2.67	0.0264	0.974045
Error	1214.36	12	101.20		

(ii) Feed

Tukey HSD test; variable Asin%
Homogenous Groups, alpha = .05000
Error: Between MS = 101.20, df = 12.000
Exclude condition: Day = 0

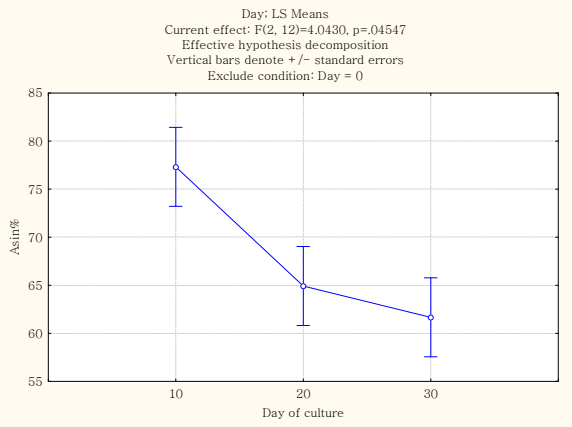
Cell No.	Feed	Asin% Mean	1
2	Live feed fed with bPOME-P	65.52907	****
1	Live feed fed with cPOME-P	70.42241	****



(iii) Day of culture

Tukey HSD test; variable Asin%
Homogenous Groups, alpha = .05000
Error: Between MS = 101.20, df = 12.00
Exclude condition: Day = 0

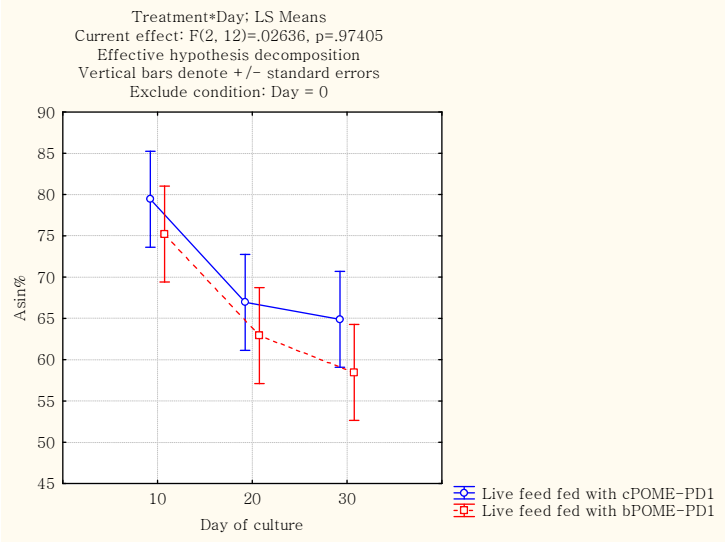
Cell No.	Day of culture	Asin% Mean	1	2
3	30	61.67341	****	
2	20	64.93018	****	****
1	10	77.32362		****



(iv) Feed x Day of culture interaction

Tukey HSD test; variable Asin%
Homogenous Groups, alpha = .05000
Error: Between MS = 101.20, df = 12.000
Exclude condition: Day = 0

Cell No.	Feed	Day of culture	Asin% Mean	1
6	Live feed fed with bPOME-P	30	58.45903	****
5	Live feed fed with bPOME-P	20	62.91711	****
3	Live feed fed with cPOME-P	30	64.88779	****
2	Live feed fed with cPOME-P	20	66.94325	****
4	Live feed fed with bPOME-P	10	75.21106	****
1	Live feed fed with cPOME-P	10	79.43618	****

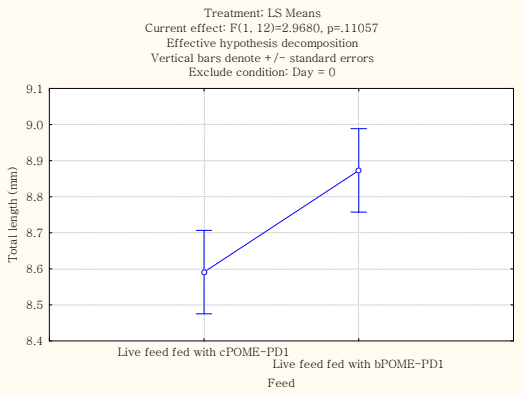


(i) Main effects (shaded area indicates significance at P < 0.01)

Effect	Univariate Tests of Significance for Total length (mm Sigma-restricted parameterization Effective hypothesis decomposition Exclude condition: Day = 0 SS	Degr. of Freedom	MS	F	p
Intercept	1372.472	1	1372.472	11395.07	0.000000
Feed	0.357	1	0.357	2.97	0.110572
Day of culture	114.533	2	57.267	475.46	0.000000
Feed*Day of culture	2.039	2	1.020	8.47	0.005093
Error	1.445	12	0.120		

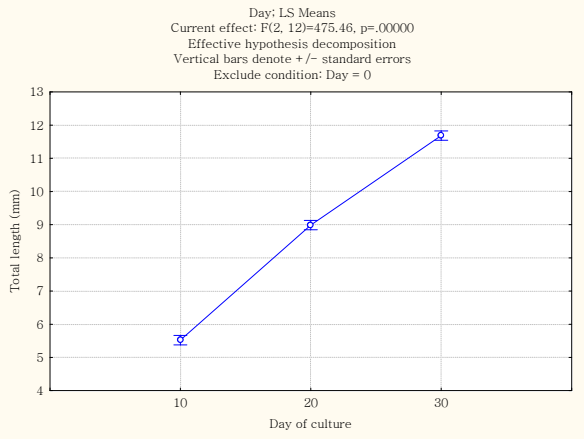
(ii) Feed

Cell No.	Tukey HSD test; variable Total length (mm) Homogenous Groups, alpha = .05000 Error: Between MS = .12044, df = 12.000 Exclude condition: Day = 0 Feed	Total length (mm) Mean	1
1	Live feed fed with cPOME-P	8.591111	****
2	Live feed fed with bPOME-P	8.872963	****



(iii) Day of culture

Cell No.	Tukey HSD test; variable Total length (mm) Homogenous Groups, alpha = .05000 Error: Between MS = .12044, df = 12.000 Exclude condition: Day = 0 Day of culture	Total length (mm) Mean	1	2	3
1	10	5.52222	****		
2	20	8.98889		****	
3	30	11.68500			****



(iv) Feed x Day of culture interaction

Tukey HSD test; variable Total length (mm)
Homogenous Groups, alpha = .05000
Error: Between MS = .12044, df = 12.000
Exclude condition: Day = 0

Cell No.	Feed	Day of culture	Total length (mm) Mean	1	2	3	4
4	Live feed fed with bPOME-P	10	5.48889	****			
1	Live feed fed with cPOME-P	10	5.55556	****			
5	Live feed fed with bPOME-P	20	8.83333		****		
2	Live feed fed with cPOME-P	20	9.14444		****		
3	Live feed fed with cPOME-P	30	11.07333			****	
6	Live feed fed with bPOME-P	30	12.29667				****